Automated Quantitative Method for Urinary Mucopolysaccharides

by

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Thesis submitted for the partial
fulfillment of the degree of

MSc in Clinical Biochemistry

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February 1996

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Acknowledgment

I thank:

Dr CS HO, my supervisor, for continuous support and advice in this project and;

Dr CP PANG, Senior lecturer of Dept. of Chemical Pathology of the Chinese University of Hong Kong, for generous donation of the urine samples from mentally retarded patients;

Dr WL MAK, Acting Senior Medical Officer in Dept. of Chemical Pathology of Prince of Wales Hospital, for generous donation of the urine samples from secondary school children.

Thanks are also given to those who have helped me to finish this project, especially the people working in the laboratory of Chemical Pathology in the Chinese University of Hong Kong and in Prince of Wales Hospital.

The following abbreviations are adopted in this study:

AB	Alcian blue
ANOVA	Analysis of variance
BM	Boehringer Mannheim
CPC	Cetypyridinium chloride
CS	Chondroitin sulphate
CTAB	Cetyl-trimethyl ammonium bromide
DMB	Dimethylmethylene blue
DS	Dermatan sulphate
GAG	Glycosaminoglycan
HPLC	High performance liquid chromatography
HS	Heparan sulphate
KS	Keratan sulphate
MPS	Mucopolysaccharidoses
SD	Standard deviation
TLC	Thin layer chromatography

Summary

This study was to develop an automated quantitative screening method for mucopolysaccharidoses(MPS). In principle, urinary glycosaminoglycans (GAGs) react with Dimethylmethylene blue (DMB) dye to form a colour complex. The change in absorbance due to a decrease of free dye concentration is monitored in an automated centrifugal analyzer. Absorbance change is related to the concentration of urinary GAG.

The spectra of DMB dye, DMB-GAG products were studied. Pure DMB had two absorption maxima: 593nm and 649nm while the DMB-GAG complexes had absorption maxima varying between 528-543nm. Quantitation of GAG concentration can be automated by monitoring the absorbance at 593nm in a Cobas Bio Centrifugal Analyzer. The DMB dye and the DMB-GAG complexes were stable for at least 300 seconds. The lowest detection limit was found to be 0.8mg/L and the linearity range was up to 70mg/L using a DMB concentration of 35 μmol/L. Sensitivity analysis gave an absorbance change of 0.285A per ug of GAG in the reaction cell. Albumin and haemoglobin did not interfere with this assay up to 5g/L and 5g/L, respectively. This assay correlated well with the CPC/Carbazole method(r=0.929). Between-batch coefficient of variation ranged from 2.4% to 6.5%. This method required a sample volume of 20μl and 5 minutes to assay for a batch of 20 samples and controls. Age specific reference range of urinary GAG/creatinine ratio for Hong Kong Chinese population was established from 545 healthy subjects with age ranging from 1 day to 70 years old. No previous reference range for Chinese subjects was reported in the literature and this was the first one. The urinary GAG/creatinine ratio was not significantly different between the two sexes. However, it was age dependent. A

group of 295 handicapped, mentally retarded patients were studied and 35% of them had elevated urinary GAG/creatinine above the 95% upper reference value.

1. Introduction

MPS are a group of inherited diseases of connective tissue metabolism. The diseases are characterized by an increased urinary excretion of GAG. Many screening and confirmation tests are available. However, current methods used are technically cumbersome and labour-intensive. Simple dye-binding screening methods using DMB has been published(1,2,3). These DMB assays measured the DMB-GAG product using absorbance values at about 530nm. Nevertheless, the absorption maxima of the complexes varied between 528-541nm and the molar absorptivity of the complexes differed(4). Thus measurement of products at one wavelength could lead to inaccurate results depending on the relative composition of different GAGs in the samples.

A recent study between interaction of the dye with GAGs has shown possible solution to this problem(4). Pure DMB dye was shown to have two absorption peaks. The alternative solution was to measure the decrease in absorbance at either one of the two peaks rather than the increase in absorbance at the product peak. This study attempts to automate the measurement of urinary GAGs in a centrifugal analyzer based on the decrease of the absorbance due to the dye.

In order to adapt this new method into routine use, reference range for urinary GAG has to be established. Reference ranges of urinary GAGs have been reported in many studies for western populations(3-6), but there is none for Asian. Therefore, this study intends to establish a reference range for local Hong Kong Chinese population. Furthermore, as part of a study on mentally retarded patients living in a care home, urinary GAG excretion in this group of patients was measured.

2. Literature Review

MPS are a group of genetic disorders characterized by the accumulation of mucopolysaccharide (glycosaminoglycan is the preferred term) in various tissues. Short life expectancy and mental retardation are found in severe forms. Presently no specific therapy is available, but supportive treatment can greatly improve the quality of life of patients. Thus, early diagnosis is helpful. It is important to design methods for its detection. In patients with MPS, increased urinary GAGs excretion is a characteristic finding. Screening methods are based on the measurement of urinary GAGs.

2.1 Properties of GAGs

GAGs are long, linear molecules made up of repeating disaccharide units, each of which contains a hexuronic acid (β-D-glucuronic or L-iduronic acid) and an amino sugar (glucosamine or galactosamine)(see Table 2.1). In keratan sulphate, galactose replaces the hexuronic acid. Most of the GAGs are highly sulphated and acetylated. They are found in relatively large amounts in skin, cartilage, bone, cornea, blood vessels, heart valves and tendons. Smaller quantities occur in liver, brain, leukocytes and mast cells(7).

Table 2.1—The disaccharide units of different GAGs

GAG	**Disaccharide Unit**
Heparan sulphate	iduronic acid/glucuronic acid and glucosamine
Dermatan sulphate	iduronic acid/glucuronic acid and galactosamine
Keratan sulphate	galactose and glucosamine
Chondroitin sulphate	glucuronic acid and galactosamine
Hyaluronic acid	glucuronic acid and glucosamine

2.1.1 FUNCTION OF GAGs

The major structural component of cartilage, proteoglycans are made up of GAGs and protein core(7). They confer resiliency to cartilage. It has been suggested that one role of proteoglycans is to prevent calcification of cartilage. Proteoglycans are found to be associated with fibers of considerable tensional strength in tissues such as tendon, deep dermis and heart valve. GAGs may have an important lubricative function in joint fluid.

2.1.2 METABOLISM OF GAGs

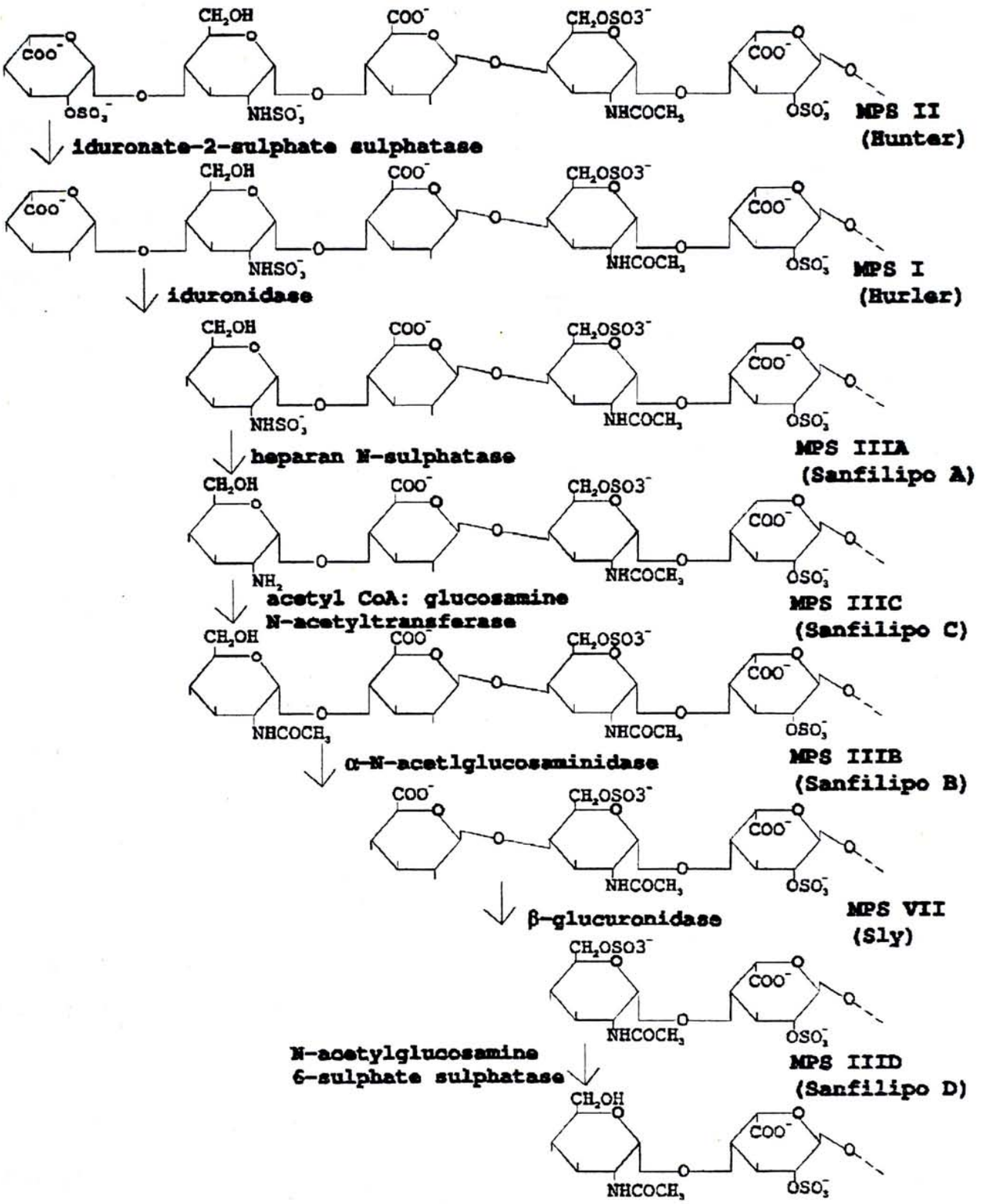
2.1.2.1 Synthesis

The sulphated GAGs in cartilage and connective tissue occur primarily as polysaccharides linked to protein. In cartilage proteoglycan biosynthesis, GAG chain initiation is a key point in regulation (7). It is dependent on the availability of core protein sites for chain initiation. A branching enzyme is needed to initiate a GAG branch from the core. Agents which suppress the level of activity of the branching enzyme would inhibit GAG synthesis. Glycosyl transferases are required for the building up of the growing chain.

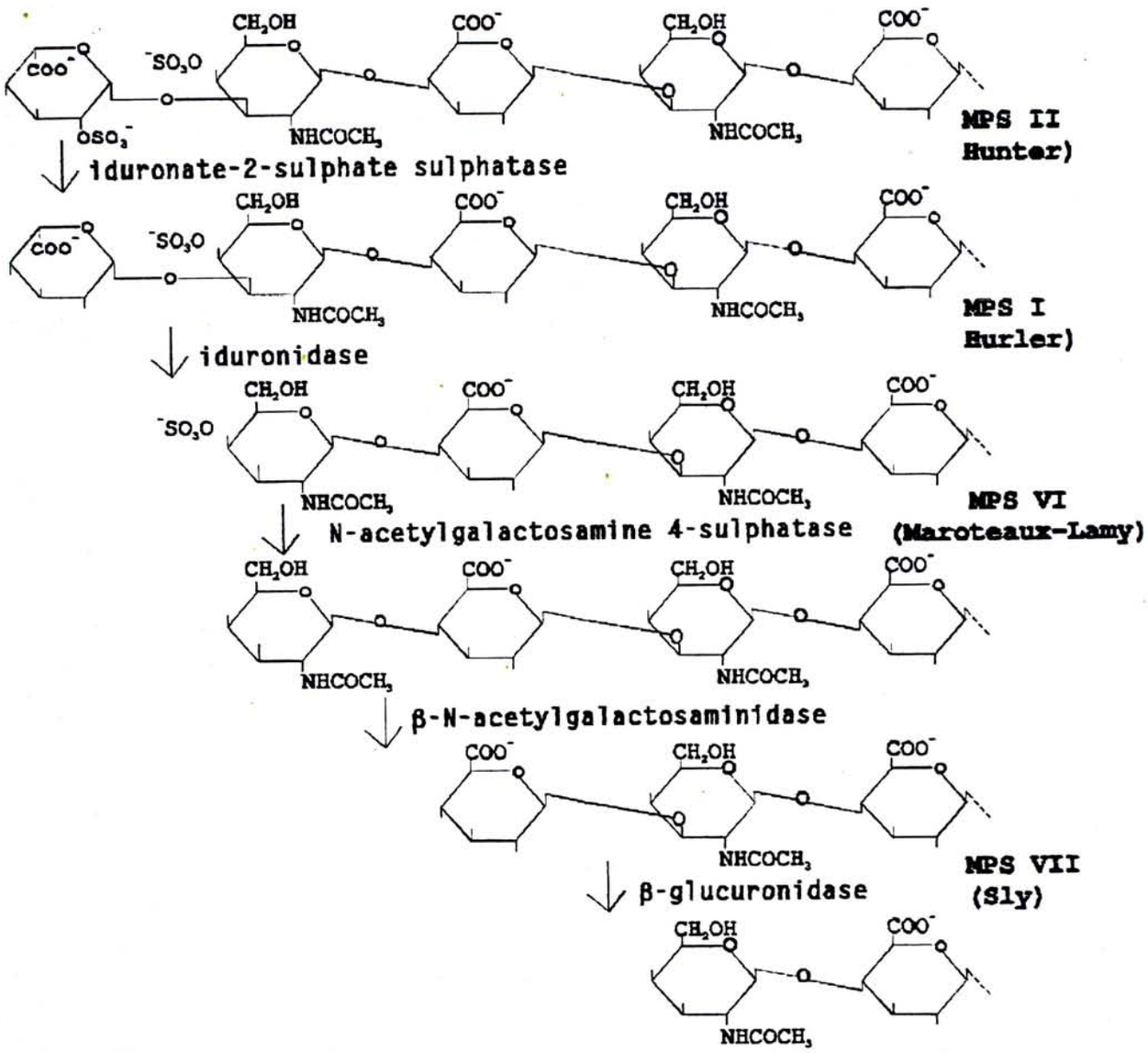
2.1.2.2 Catabolism

The GAGs are degraded primarily by lysosomal enzymes that sequentially cleave a monosaccharide or sulphate from the non-reducing terminus of the mucopolysaccharides chain. At least eleven enzymes including four glycosidases, five sulphatases and one nonhydrolytic transferase (9) are required to degrade the mucopolysaccharide chain (8). Defects of any one of these enzymes interrupt the sequence of degradation, resulting in the mucopolysaccharides chain being resistant to further cleavage by the enzymes. It will result in the accumulation of GAGs in the lysosomes. Figures 2.1, 2.2, 2.3 depict the enzymology of lysosomal degradation of dermatan sulphate, heparan sulphate and keratan sulphate and the enzyme deficiencies in the MPS. Table 2.2 summarizes the enzyme deficiencies reported in MPS and the major storage substances reported for theirs disorder based on the McKusick's and Neufeld Classification (9).

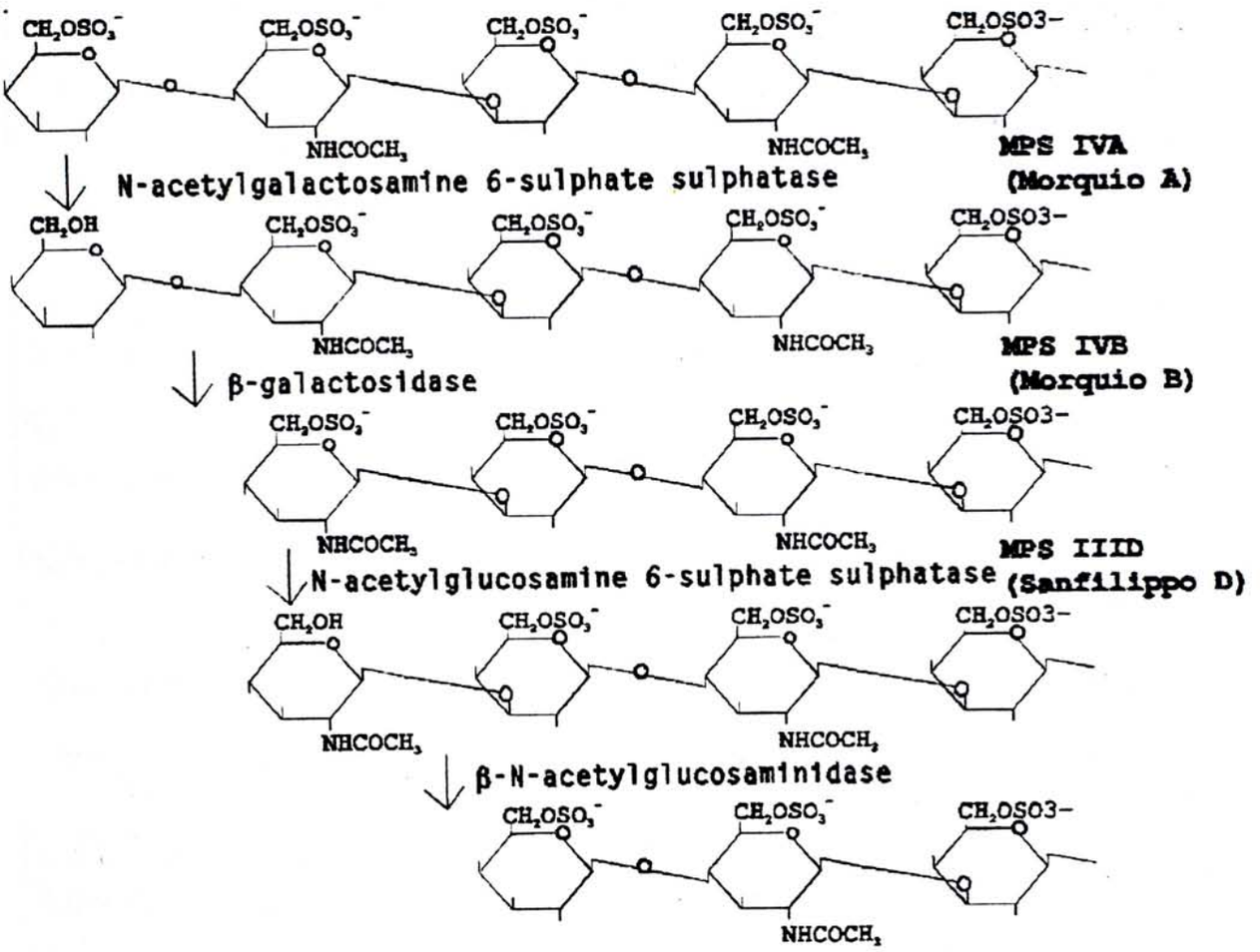
There are two types of lysosomal glycosidases: exoglycosidases and endoglycosidases. Exoglycosidases hydrolyze linkages only at the non-reducing terminus of the GAGs. Endoglycosidases cleave interchain linkages. The missing enzymes that lead to MPS are exoglycosidases. In some tissues, GAGs can undergo limited degradation by endoglycosidases. The incompletely degraded GAG fragments then accumulate in the tissues or are excreted in the urine.



Figures 2.1—Degradation of heparan sulphate and the diseases caused by deficiency of specific enzymes



Figures 2.2—Degradation of dermatan sulphate and the diseases caused by deficiency of specific enzymes



Figures 2.3—Degradation of keratan sulphate and the diseases caused by deficiency of specific enzymes

TABLE 2.2—The classification, enzyme deficiency and urinary GAG pattern in different kinds of the MPS(9)

Number	Eponym	Enzyme Deficiency	Urinary GAGs
MPS I-H	Hurler	α-L-Iduronidase	Dermatan sulphate, heparan sulphate
MPS I-H/S	Hurler/Scheie	α-L-Iduronidase	Dermatan sulphate, heparan sulphate
MPS I-S	Scheie	α-L-Iduronidase	Dermatan sulphate, heparan sulphate
MPS II-A	Hunter, severe	Iduronate sulphatase	Dermatan sulphate, heparan sulphate
MPS II-B	Hunter, mild	Iduronate sulphatase	Dermatan sulphate, heparan sulphate
MPS III-A	Sanfilippo A	Heparan N-sulphatase	Heparan sulphate
MPS III-B	Sanfilippo B	α-N-acetylglucosaminidase	Heparan sulphate
MPS III-C	Sanfilippo C	Acetyl coenzyme A: α-glucosamine-N-acetyltransferase	Heparan sulphate
MPS III-D	Sanfilippo D	N-acetyl-α-glucosamine-6-sulphatase	Heparan sulphate
MPS IV-A	Morquio A	N-acetylgalactosamine-6-sulphatase	Keratan sulphate
MPS IV-B	Morquio B	β-galactosidase	Keratan sulphate
MPS VI	Maroteaux-Lamy	N-acetylgalactoasamine-4-sulphatase (arylsulphatase B)	Dermatan sulphate
MPS VII	Sly	β-glucuronidase	Dermatan sulphate, heparan sulphate, chondroitin sulphates

2.2 *Mucopolysaccharidoses*

MPS are a group of inherited lysosomal storage diseases caused by deficiency of specific lysosomal enzymes which are needed for the stepwise degradation of GAGs (9). In 1917, Hunter (10) presented a description of two brothers who appeared to have had the sex-linked MPS which bear his name. In 1919, Gertrude Hurler (11) described two unrelated boys with coarse facial features, multiple skeletal abnormalities, corneal clouding, hepatosplenomagaly and cardiac involvement. This detailed description served as the prototype for the descriptions of MPS that followed. In 1952, Brante (12) introduced the term 'mucopolysaccharidoses' based on the finding of excessive GAG accumulation in the liver of a patient with the Hurler syndrome. In 1957, Dorfman and Lorincz(13) discovered the excessive secretion of GAG in the urine of Hurler syndrome. In 1965, McKusick (14) systematized the classification of the MPS based on the types of GAGs excreted in the urine, mode of inheritance and clinical features.

2.2.1 CLINICAL PRESENTATION AND GENETICS OF MPS

2.2.1.1 Clinical manifestation

MPS are progressive in nature and involve multiple tissues and organs. The MPS share many clinical features, including neurological, visceral and skeletal disorders. Organomegaly, dysostosis multiplex and abnormal facies are some common characteristics. Hearing, vision, cardiovascular function and joint mobility may be affected. Table 2.3 summaries the clinical manifestations, and onset of the diseases (9).

2.2.1.2 Genetics and incidences

The incidence of MPS are rare. MPS type III has the highest incidence(1 in 24000). Most of them are inherited as autosomal recessive. MPS type II is an exception, which is sex-linked. The genetics and incidences of the diseases are listed in the table 2.4.

2.2.2 Treatment

Presently no specific treatment is available. Management of MPS patients consists of supportive care and treatment of complication. In diseases (MPS IS and MPS VI) where life expectancy and intelligence are not affected, management of the clinical symptoms seems indicated such as treatment of the corneal clouding, the aortic valve disease (7,15).

In severe diseases (e.g. MPS II), therapeutic trials with 'correcting factors'(shown to be lysosomal enzymes) (7), fibroblast transplantation (16), amnion transplantation (17) and bone marrow transplantation have been performed. Bone marrow transplantation(BMT) is promising. There are a number of reports evaluating the efficacy of BMT for the treatment of MPS: MPSI (18-20), MPSII (21), MPSVI (22,23). Treatment by gene transfer is under research (9).

TABLE 2.3—The clinical manifestations, and onset of the diseases in different kinds of the MPS(7,9,24)

Number	Clinical manifestations	Year of onset
MPS1H	Corneal clouding, dysostosis multiplex, organomegaly, heart disease, mental retardation, death in childhood	6-12 months, death usually before age 10
MPS1S	Corneal clouding, stiff joints, normal intelligence and life span	5-15 years
MPS1H/S	Phenotype intermediate between 1H and 1S	2-4 years, survival into 20s
MPSII(severe)	Dysostosis multiplex, organomegaly, no corneal clouding, mental retardation	2-4 years, death by 10-15 years
MPSII(mild)	Normal intelligence, short stature	first decade, survival to 20s to 60s
MPSIIIA	Profound metal deterioration, hyperactivity, relatively mild somatic manifestations	2-6 years, death at end of puberty
MPSIIIB MPSIIIC MPSIIID	Clinically indistinguishable from MPSIIIA Phenotype similar to IIIA Phenotype similar to IIIA	onset later than IIIA, longer survival than IIIA (24)
MPSIVA	Distinctive skeletal abnormalities, corneal clouding, odontoid hypoplasia, milder forms known to exist	1-3 years (9), death by 20-40 years (7)
MPSIVB	Short stature, corneal clouding, mild dysostosis multiplex, prominence of lower face, pectus carinatum, hip deformity, normal intelligence	onset later than IVA (9)
MPSVI	Dysostosis multiplex, corneal clouding, normal intelligence, milder forms known to exist	4 years, death in 20s
MPSVII	Dysostosis multiplex, hepatosplenomegaly, wide spectrum of severity	1-2 years

TABLE 2.4— The genetics and incidences of the MPS(15,25-29)

Number	Incidence	Genetics/transmission	Reference
MPS1H	1 in 100,000	Homozygous for MPS1H gene, autosomal recessive	(25)
MPS1S	1 in 500,000	Homozygous for MPS1S gene, autosomal recessive	(15)
MPS1H/S	1 in 115,000	Genetic compound for MPS1H and 1S genes, autosomal recessive	(15)
MPSII(severe)	1 in 67,500	Hemizygous for X-linked gene, sex-linked	(26)
MPSII(mild)	1 in 67,500	Hemizygous for X-linked gene, sex-linked	(27)
MPSIIIA MPSIIIB MPSIIIC MPSIIID	1 in 24,000	Homozygous for Sanfilippo A gene, autosomal recessive Homozygous for Sanfilippo B gene, autosomal recessive Homozygous for Sanfilippo C gene, autosomal recessive Homozygous for Sanfilippo D gene, autosomal recessive	(27)
MPSIVA MPSIVB	1 in 300,000	Homozygous for Morquio A gene, autosomal recessive Homozygous for Morquio B gene autosomal recessive	(28)
MPSVI	rare, no data	Homozygous for allele at M-L locus, autosomal recessive	(26)
MPSVII	fewer than 20 cases described	Homozygous for mutant gene at β-glucuronidase locus, autosomal recessive	(29)

2.3 *Laboratory tests for the diagnosis of MPS*

2.3.1 SCREENING

Measurement of GAG content in urine is generally used as screening procedure for MPS. Infected samples should not be used (5). Some preservatives may interfere with the analyses e.g. toluene lowers the efficiency of precipitation methods (5). Samples are to be frozen solid as soon as possible after collection.

2.3.1.1 Spot test

Paper spot tests are based on the interaction of urinary GAGs with a suitable cationic dye such as toludine blue (30), alcian blue (AB)(31,32), azur A (33). A fixed volume of urine is applied to filter paper, dried and stained with a solution of the dye. Spot tests are easy to perform. However, they are either qualitative or semi-quantitative only. High incidence of false-negative results was reported (5). Thus paper spot tests are not recommended as screening tests for MPS.

2.3.1.2 Turbidity test

It is based on the precipitation of GAGs with acidified albumin (34,35), quaternary ammonium compounds such as cetyl-trimethyl ammonium bromide(CTAB) (36), cetypyridinium chloride(CPC) (37), and CPC citrate test (38). Precipitation of GAGs with CPC or CTAB is affected by ionic strength and pH (39) as well as by concentration. If these factors are not taken into account, urine samples may give false positive or false negative results (5). In a method described by Pennock (5), the effects of ionic strength and pH could be overcome by buffering the CPC solution with citrate at pH 4.8.

2.3.1.3 Uronic acid-carbazole test

Quantitative estimation is based on measurement of hexuronic acid residues which all GAGs contain, except keratan sulphate. Before quantitation, it has to carried out a suitable GAG preparation. The preparation is to separate GAGs from other interfering substances and can be achieved by: dialysis (40,41); dialysis followed by fractionation on cellulose columns (42,43); precipitation with CTAB, CPC (44,45) or aminoacridine (46,47). The hexuronic acid in the GAGs is measured by reaction with the carbazole-sulphuric acid (48). The hexuronic acid will react with carbazole in the presence of concentrated sulphuric acid to give a magenta coloured product. Quantitative determination of the hexuronic acid residues of GAGs with the carbazole-sulphuric acid reagent has the disadvantage that it fails to detect the keratan sulphaturia of Morquio's syndrome(MPS IV). In addition the assay is potentially dangerous, owing to the use of concentrated sulphuric acid.

2.3.1.4 Dye binding

It is based on the direct interaction of GAGs with metachromatic dyes such as AB(49,50) and DMB(1,51). It allows automation in routine chemical analyzers and will be discussed later. Gold (50) described a method based on AB for the direct measurement of GAGs on untreated urine samples. Recently, de Jong et al (49) described a modified AB method to estimate GAGs in the presence of other polyanions (52) and compared their method with DMB and the results were summarised on table 2.5.

Table 2.5 Comparison between modified AB and DMB.

	modified AB	**DMB**
Sample volume	180ul	5-20ul
CV(10 runs)	11%	4%
Recovery(without protein) (with protein)	78-95% 76-111%	94-103% 96-100%
Correlation(non MPS) (MPS)	AB=1.055DMB-0.029, r=0.861 AB=1.126DMB+6.225, r=0.928	
Standard deviation scores	8.5	18.2

The DMB assay had a higher sensitivity and the smaller sample volume used probably made it less sensitive to interfering substances. It also showed a better discriminatory power between MPS and normal urine samples. Therefore DMB assay was better suited for urinary screening for MPS than the AB assay.

However, the DMB assays developed so far involved the measurement of the dye-GAG complex. The different reaction products of the dye-GAG complex show different molar absorptivity, making spectrophotometric quantitation difficult. A recent study (4) of the interaction of the dye with GAG has shown possible solution to this problem by measuring the decrease of the absorbance due to the dye, not the increase of absorbance due to the product.

2.4 Confirmation method

Confirmation method can be made either by identifying the missing enzyme in serum or the accumulated GAGs in urine. The enzymes essential for the catabolism of GAGs are shown in the figures 2.1, 2.2, 2.3. The accumulated GAG products in urine characteristic of each MPS are shown in table 2.2.

2.4.1 Enzyme assay

Enzyme assay is specific and reliable. Definitive diagnosis of the MPS is established by enzyme assays (53,54). Samples such as cultured fibroblasts may be used for all the MPS; leukocytes for most MPS; serum or plasma for MPS I, II, IIIB and VIIA. The enzyme assay can be performed using chromogenic, fluorigenic or radioactive substrates.

Prenatal diagnosis is possible for all the MPS. Samples include amniotic fluid, cultured cells or uncultured cells from amniotic fluid, chorionic villus biopsies. Carrier detection for the autosomal recessive MPS is possible by measuring the relevant enzyme in leukocytes or serum. Statistically heterozygotes show activity half of the normal level. The wide range and the overlap between the heterozygotes and normal groups make classification of a particular individual difficult and at times impossible. They may be used to determine the carrier status of relatives of affected patients who are at risk but are not practical for large scale screening. Carrier detection in sex-linked Hunter's syndrome had been achieved by cloning of cultured fibroblasts (55,56) or testing hair roots(57,58) but these methods are labor-intensive and costly.

2.4.2 Electrophoresis

Sulphated GAGs can be separated by electrophoresis on a cellulose acetate membrane in barium acetate buffer (59) or barbitone buffer (37). The separation is based on the differences of GAG structure. Electrophoresis in barbitone buffer cannot separate dermatan sulphate from chondroitin sulphates, nor heparan sulphate from keratan sulphate (5). However, it is a useful method for separating galactosamine containing GAG from glucosamine containing GAGs. Electrophoresis in barium acetate gives good separation of dermatan sulphate and heparan sulphate (60) but give poor separation between keratan sulphate and chondroitin sulphates.

2.4.3 Chromatography

TLC is used to separate the GAGs according to their solubility in different solvents. Many methods have been described (61,62). The problems associated with these methods include difficulty in separating dermatan sulphate (61) or keratan sulphate (62) from other GAGs. A method is based on the relative solubility of different GAGs, as their calcium salts, in different concentration of alcohol (63,64).This method was claimed to clearly separate keratan sulphate from other GAGs and was thus useful in the diagnosis of Morquio's disease. However, this method involves the use of running a TLC plate in six separate solvents in sequence. Certainly it is tedious and time-consuming. One study also investigated the application of HPLC in the identification and quantitation of GAGs (65).

2.5 Conclusion of Literature Review

1. Paper spot tests are qualitative or semi-quantitative only and give high incidence of false-negative results (5).

2. Quantitative determination of the hexuronic acid residues of GAG molecules with the borate-carbazole reagent fails to detect the keratan sulphaturia of Morquio's syndrome(MPS IV).

3. Many existing methods depend on the isolation of GAGs by precipitation with quaternary ammonium salts or on separation techniques such as electrophoresis (5,59) or TLC (61-64). These methods are technically cumbersome, labour-intensive and require large urine specimens (1 to 10 ml).

4. Precipitation of GAGs with CPC or CTAB is affected by ionic strength and pH (39) as well as by concentration. If these factors are not taken into account, urine samples may give false positive or false negative results (5).

5. Enzyme assays are specific and reliable. But they are labour-intensive and not convenient for screening.

6. Existing methods are not practicable for mass screening because of

   - the large sample volume required,
   - preparative isolation procedures required before quantitation,
   - interference by other negatively charged ions other than sulphated GAG in direct assay (50,66).

2.6 *Choice of method*

1. Knowledge of the urinary GAG concentration coupled with the clinical manifestation can help to choose the most appropriate enzymatic assay to diagnose conclusively the specific type of MPS.

2. MPS disease is presented clinically between 9 months and 4 years of age and detection of increased GAGs in urine may confirm the diagnosis.

3. With the recent progress in the treatment of these disorders, earlier therapeutic intervention is crucial. Therefore presymptomatic diagnosis by means of routine mass screening of all newborn infants is strong motivation.

4. The enzymatic determinations are often tedious and expensive and must be preceded by the study of GAGs excreted in urine.

2.7 *The present study*

This study intend to investigate the spectra of the pure DMB, individual GAG reacting with DMB, and mixtures of GAGs reacting with DMB. A suitable wavelength for the quantitation of GAGs is to be evaluated. The peak wavelength or nearby wavelength of DMB will be used to quantify GAGs if it is successful.

Then this study will investigate the assay characteristics such as,

1. linearity,
2. precision at low, median, and high range of the curve,
3. within-batch, between-batch, and day-to-day precision,
4. detection limit,
5. correlation with a standard method,
6. carryover study,
7. interference study.

Reference ranges are set up by measuring samples from reference populations. The capability of the assay to detect the disease is investigated by measuring known patient samples. Furthermore, mentally retarded patients were also investigated.

3. Materials

3.1 Material

Formic acid sodium salt, tris(hydroxymethyl)aminomethane (Tris), chondroitin sulphate C sodium salt, dematan sulphate sodium salt (chondroitin sulphate B), heparan sulphate sodium salt, keratan sulphate sodium salt were purchased from Sigma (St. Louis, MO, USA). 1,9-dimethyl-methylene blue was bought from Aldrich Chemical Company (Milwaukee, WI, USA). Formic acid and absolute ethanol were bought from E. Merck(64271 Darmstadt, Germany). Serum bovine albumin was brought from Othro-Diagnostic (Raritan, NJ, USA).

3.2 Stock solution

Solution A—DMB was prepared by dissolving 34.8mg in 5ml 95% ethanol.

Solution B—Formate buffer(55mmol/L) was prepared by mixing 3.74g sodium formate with distilled water and 2ml formic acid and diluted to 1L distilled water.

Solution C—It was prepared by mixing 5ml solution A with 995ml solution B. The final concentration of DMB was 100μmol/L, formate buffer 55mmol/L, pH3.3.

Solution D—Tris(hydroxymethyl)-aminomethane (2mol/L) was prepared by dissolving 242.2g Tris in 1L distilled water.

3.3 Stock standards

Chondroitin sulphate(CS) was prepared by dissolving 1mg CS in 10ml distilled water. Dermatan sulphate(DS), Heparan sulphate(HS) and Keratan sulphate(KS) were similarly prepared. The concentration of the four standard solutions were 100mg/L.

3.4 Working standards

Working standard solutions of CS, DS, HS and KS (80, 60, 40, and 20 mg/L) were prepared by diluting the stock standards appropriately.

3.5 Working DMB solution

Freshly prepared working DMB solution(DMB concentration: 30μmol/L, Tris buffer 0.2mol/L, pH8.8) was prepared by mixing 5.5ml of solution B, 3.5ml of solution C and 1 ml of solution D.

3.6 Instruments

Gilford Response UV-VIS spectrophotometer was from Gilford Instrument Laboratories(Oberlin, OH, USA). Cobas Bio Centrifugal Analyzer was from Hoffmann-La Roche (Basle, Switzerland). Hitachi Automatic Analyzer Model 911 was from Hitachi Ltd (Tokyo, Japan).

3.7 Statistics

Analysis of variance(ANOVA) was used to check for linearity between the curves of DMB-GAG reaction. Significance level was set at 0.05. Student t-test was used to check for sex difference in reference range study. Significance level was set at 0.05. Mann Whitney test was used to test for significance difference between sex in the same age groups. The reference range was obtained by ranking the data and taking values between 2.5^{th} percentiles to 97.5^{th} percentiles. Other statistical methods such as mean, standard deviation, coefficient of variation were analyzed by personal computer package, Excel version 5 (Microsoft Corporation, USA).

3.8 Urine samples

In this study, urine samples of apparent healthy Chinese subjects were collected from neonates in hospital nursery wards, laboratory staff, secondary school children for check-up, patients in the out-patient department to establish the reference range. Urine samples of mentally retarded patients were from a care home in the Caritas Medical Center. Urine samples of MPS patients were from the Prince of Wales Hospital. MPS patients were diagnosed on the basis of clinical symptoms and enzyme deficiencies in leukocytes and/or fibroblasts. All the urine specimens were either analyzed promptly or stored at -70°C until assay.

4. Methods

4.1 Studies on spectral characteristics

4.1.1 Spectrum of DMB

Working DMB solution was freshly prepared. The solution mixture was scanned over the wavelength range of 450-800nm in a Gilford Response UV-VIS spectrophotometer.

4.1.2 Spectra of DMB-GAG products

80µl of 60mg/L CS was added to 1ml of freshly prepared DMB working solution and mixed. The solution was added to the cuvette of the spectrophotometer immediately. A spectrum was obtained by scanning the solution over the wavelength range of 450-800nm. Similarly the spectra of DMB-DS(DS, 70mg/L), DMB-HS(HS, 80mg/L) and DMB-KS(KS, 50mg/L) were obtained.

4.2 Studies on stability

4.2.1 Stability of DMB dye in assay condition

The stability of the DMB working solution was investigated in a Cobas Bio Centrifugal Analyzer. The absorbance of the pure dye at wavelength of 593nm was monitored for 5 minutes at an interval of 10 seconds. The instrument settings for Cobas Bio were given in appendix 1.

4.2.2 Stability of DMB-GAG products in assay condition

The stability of the DMB-GAG reaction products was studied using the Cobas Bio Centrifugal Analyzer. 20µl of a 60mg/L of CS working solution were added to the cuvette rotor of the analyzer. After an initial 10 seconds incubation time, the absorbance was measured at 593nm for 5 minutes at an interval of 10 seconds. Similarly the stability of DMB-DS, DMB-HS and DMB-KS were also studied. The instrument settings for Cobas Bio was the same as given in appendix 1 except that sample volume was 20µl and incubation time was 10s.

4.3 Linearity studies

4.3.1 Linearity of DMB dye

Different concentrations of DMB solution were obtained by making appropriate dilutions of buffer solution B with D according to the ratio shown in table 4.1. The absorbance of the different concentrations of DMB solution was monitored in the Cobas Bio Centrifugal Analyzer. The instrument settings for Cobas Bio were given in appendix 2.

Table 4.1 Preparation of DMB solutions

Solution B, ml	Solution C, ml	Solution D, ml	Concentration of DMB in final mixture (μmol/L)
0	9.0	1.0	90
2.0	7.0	1.0	70
4.0	5.0	1.0	50
4.5	4.5	1.0	45
5.0	4.0	1.0	40
5.5	3.5	1.0	35
6.0	3.0	1.0	30
6.5	2.5	1.0	25
7.0	2.0	1.0	20
7.5	1.5	1.0	15
8.0	1.0	1.0	10
9.0	0	1.0	0

4.3.2 Linearity of GAG assay

Standard solutions of CS, DS, HS and KS (80, 60, 40, 20, and 0mg/L) were allowed to react with working DMB solution. The reaction was monitored in the Cobas Bio Centrifugal Analyzer using the same instrument settings as given in appendix 2 except that sample volume was 20μl. The change in absorbance (ΔA) was plotted against the concentration of GAGs.

4.4 Detection limit

Detection limit was determined by assaying a saline sample(zero concentration of GAG) for 30 times. The mean ΔA and standard deviation were calculated. The mean plus 3SD of ΔA value was calculated and the corresponding lower detection limit in mg/L was read from the calibration curve of the assay.

4.5 Precision study

4.5.1 Within-run precision

Quality control samples were prepared by spiking known amounts of HS in a urine sample of low GAG concentration to make a high control (about 45mg/L) and a low control (about 10mg/L). The GAG concentrations of 20 consecutive cups of the 2 levels of controls were determined in the Cobas Bio Centrifugal Analyzer. The mean and standard deviation for each level were calculated.

4.5.2 Between-day precision

The high and low controls prepared in section 4.5.1 were assayed each day for 20 days to determine the between-day precision.

4.6 Interference study

4.6.1 Haemoglobin

Haemoglobin from washed erythrocytes was lysed by distilled water to give a concentration of 10g/L. Other concentrations of the haemolysate were obtained by further dilution of this solution with appropriate amount of distilled water. Equal volume of known concentration of haemolysate was added to equal volume of HS standard solutions to make a final haemoglobin concentration ranging from 0 to 5g/L and a GAG concentration of 20mg/L. The samples with and without haemolysate addition were assayed using working DMB solution. The differences in concentration were calculated.

4.6.2 Protein

The influence of protein on the DMB assay was studied by adding protein to the HS standards and urine samples. Equal volume of known concentration (e.g. 10g/L) of serum bovine albumin was added to equal volume of HS standard solutions to make a final protein concentration from 0 to 5g/L and a GAG concentration of 35mg/L. The apparent concentration of samples with and without protein addition in pairs was determined using DMB solution. The difference in concentration was calculated.

4.7 Recovery study

HS standard solutions (e.g. 10, 25 and 50mg/L) were added to equal volume of a urine sample. Same volume of distilled water was added to the urine sample to provide a baseline sample. GAG concentration in the test and baseline samples were analyzed in triplicates within-batch. The recovery ratio was calculate as follows:

$$\% \text{ recovery} = 2X(R_{test} - R_{base})/C_{standard} X100$$

where R_{test}= result of the test sample

R_{base}=result of the baseline sample

$C_{standard}$= Concentration of standard

4.8 Correlation study

The new DMB method was compared with the conventional procedure of CPC/Carbazole reaction(48). Urine samples extending over the range of normal and pathological GAG concentrations were analyzed by both methods. Forty urine samples were collected from normal individuals and from MPS patients. The results were expressed in relation to creatinine concentration of the urine samples. Carbazole assay was performed as described by Pennock(5) and is routinely used in the Department of Chemical Pathology at the Prince of Wales Hospital.

4.9 Automated measurement of urinary GAG

Urinary GAG was measured in the Cobas Bio Analyzer using instrument setting as given in appendix 2 except that sample volume was 20μl and standard concentrations were HS 20, 40, 60 mg/L. Urinary creatinine was measured by kinetic Jaffe reaction in a Hitachi 911 Analyzer using urinary mode and BM-cfas standard. All results were expressed in mg GAG/mmol creatinine ratio.

4.10 Reference range

Apparent healthy Chinese subjects were recruited from neonates in hospital nursery wards, laboratory staff, secondary school students for check-up, patients in out-patient department. All the subjects were checked to have no clinical history of renal and liver diseases, nor inherited disorders. Untimed urine specimen were collected from the reference subjects and the GAG content were measured. Then Reference range was obtained by partitioning the reference subjects into a number of age groups according to de Jong el at(6):

- 0—5 months
- 6—12 months
- 13—23 months
- 2—3 years
- 4—5 years
- 6—7 years
- 8—9 years
- 10—14 years
- 15—19 years
- >20 years

GAGs excretion is age dependent. Excretion was highest in neonates and decreased with age. Hence more partitions were found in young children.

4.11 GAG in mentally retarded patients

Urine samples from a population of mentally retarded patients in Caritas Medical Center were analyzed for their urinary GAG excretions. The result was expressed in amount of GAG in relation to urine creatinine excretion. Individual result was then compared with the appropriate age-related reference range established in section 4.10.

5. Results

5.1 Studies on spectral Characteristics

5.1.1 DMB Spectrum

The spectrum of pure DMB dye was shown in figure 5.1. The pure dye had two absorption peaks at 593 and 649nm.

5.1.2 Spectra of DMB-GAG products

The spectrum of pure DMB dye and the spectra of the DMB-CS, DMB-DS, DMB-HS and DMB-KS products were shown as a composite in figure 5.2. Each DMB-GAG spectrum of the had three peaks: the first peak belonged to the DMB-GAG complex; the second and the third peaks belonged to the DMB dye. The absorbance maxima of these peaks were given in table 5.1.

Table 5.1 Absorption maxima of DMB-GAG products

GAG	First peak, nm	Second peak, nm	Third peak, nm
CS	527.5	594	650
DS	530.5	593.5	649
HS	540.5	593	650
KS	540.5	593	649

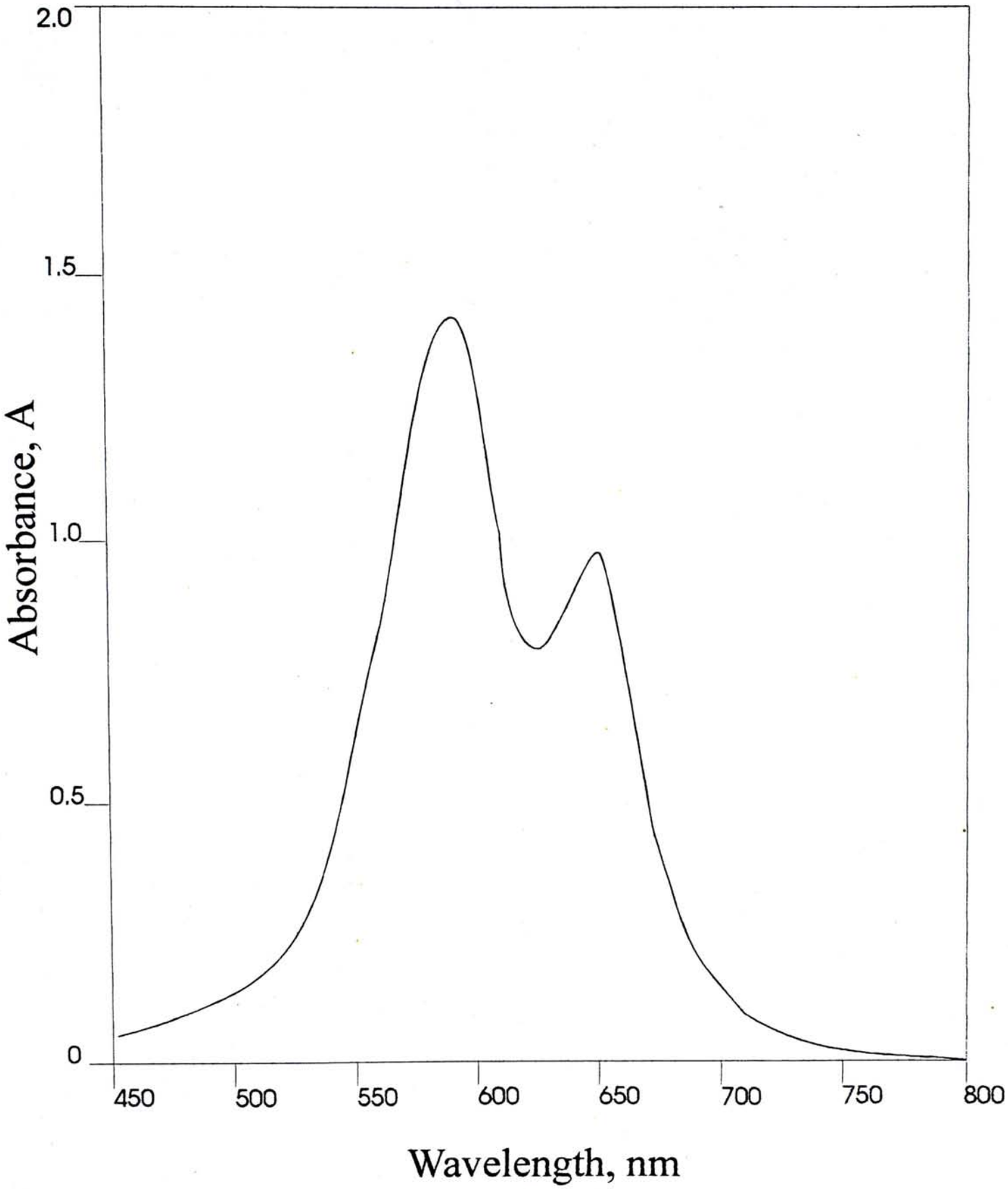


Figure 5.1 Spectrum of DMB Dye

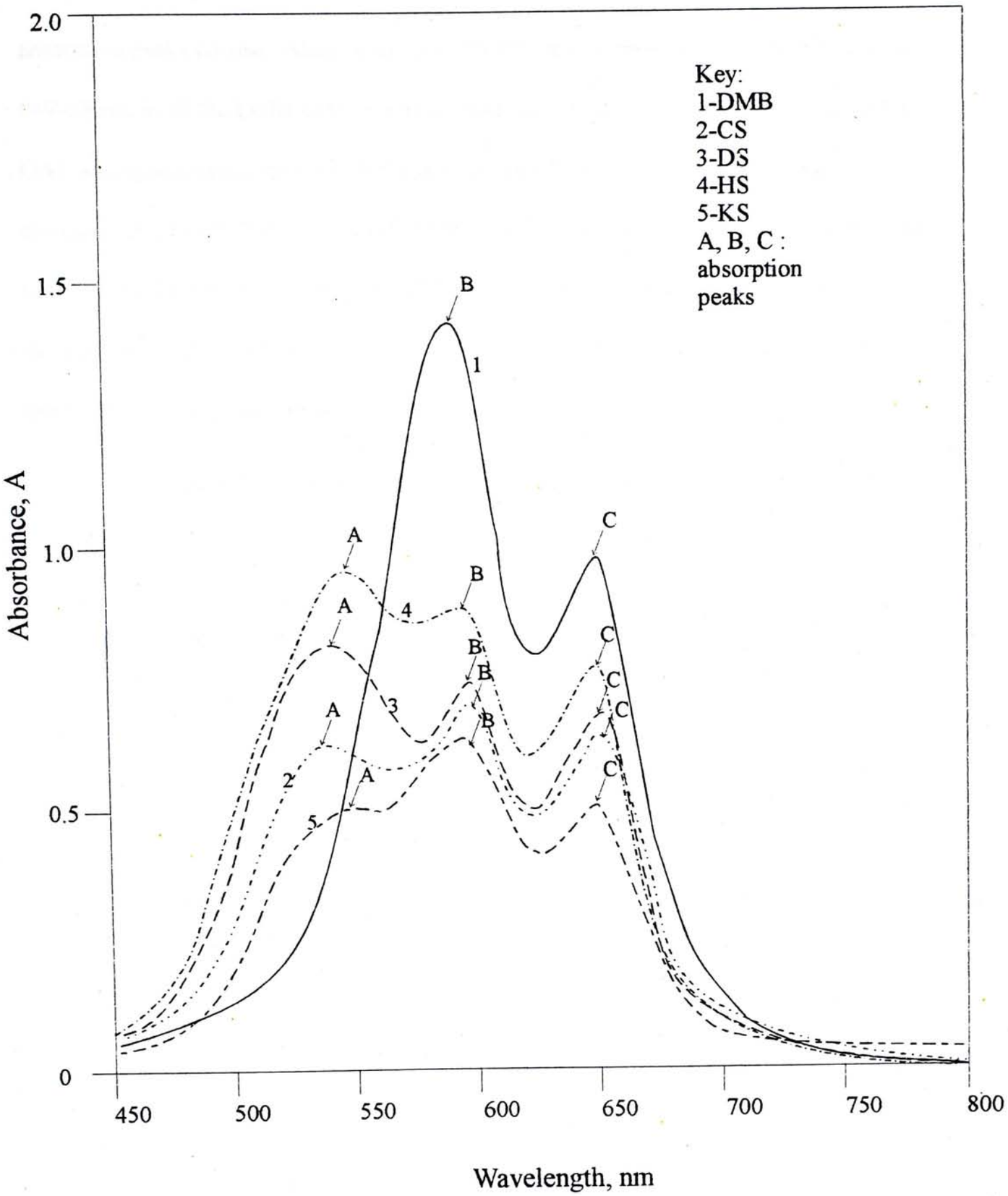


Figure 5.2 Spectra of DMB and DMB-GAG complex

The absorption maxima of the DMB-GAG products ranged from 528-541nm. The absorption peaks corresponding to the pure DMB solution were found to be 593-594 and 649-650nm in all the DMB-GAG reaction mixtures. These results showed that the DMB-GAG absorption peaks varied with the type of the GAGs. However, the absorption peaks corresponding to DMB dye remained at the same wavelengths. It is not desirable to assay GAGs using the first absorption peak of the complexes as the peak wavelength varies with the different kind of GAGs. It is better to assay the GAGs by monitoring the reaction at one of the two absorption peaks of the DMB dye.

The changes of absorbance at the 3 absorption peaks for the DMB-DS reaction were plotted against DS concentration (Figure 5.3). The 593nm gave a greater response with the concentration of GAG under study.

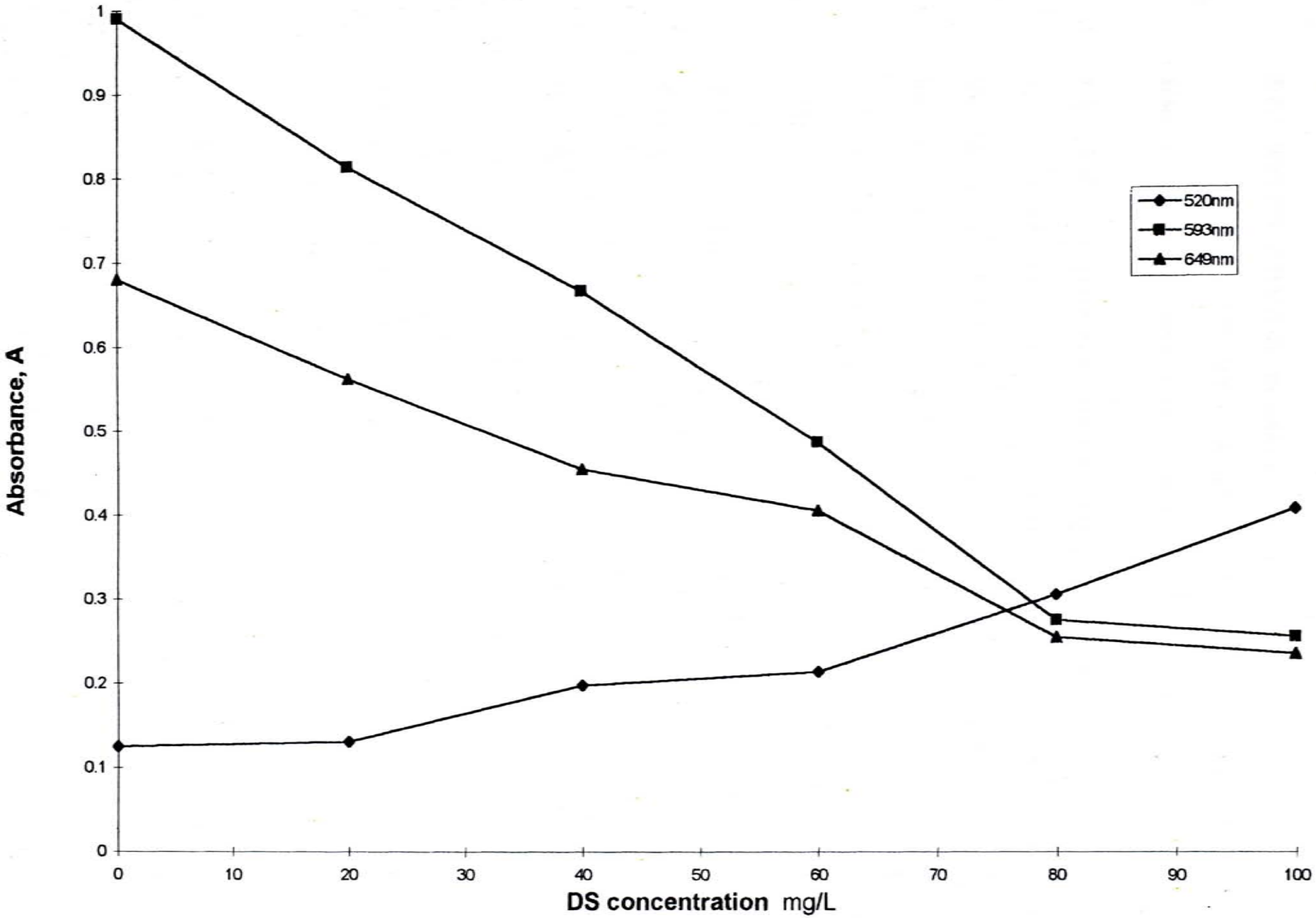


Figure 5.3 Change of absorbance at various DS concentration at three wavelengths

5.2 Studies on stability

5.2.1 Stability of DMB dye in assay condition

The change of absorbance against time for DMB dye was shown in figure 5.4. The absorbance at 593nm remained unchanged for at least 300 seconds.

5.2.2 Stability of DMB-GAG reaction mixture in assay condition

The change of absorbance of DMB-CS against time was shown in figure 5.5a. It showed that the absorbance of the reaction mixture at 593nm remained unchanged for at least 300 seconds. Similar absorbance change was also observed for DMB-DS, DMB-HS and DMB-KS as shown in figure 5.5b-5.5d. A time interval of 40 seconds was chosen to assay the GAGs.

5.3 Linearity studies

5.3.1 Linearity of DMB dye

The absorbance of the different concentrations of DMB solutions was monitored in the Cobas Bio Centrifugal Analyzer. Figure 5.6 showed the change in absorbance versus DMB concentrations. It was found that the curve was linear up to the DMB concentration of 40μmol/L. DMB concentration of 35μmol/L was used in the GAG assay. Linearity response of the Cobas Bio Centrifugal Analyzer is up to 3.0A at 340nm.

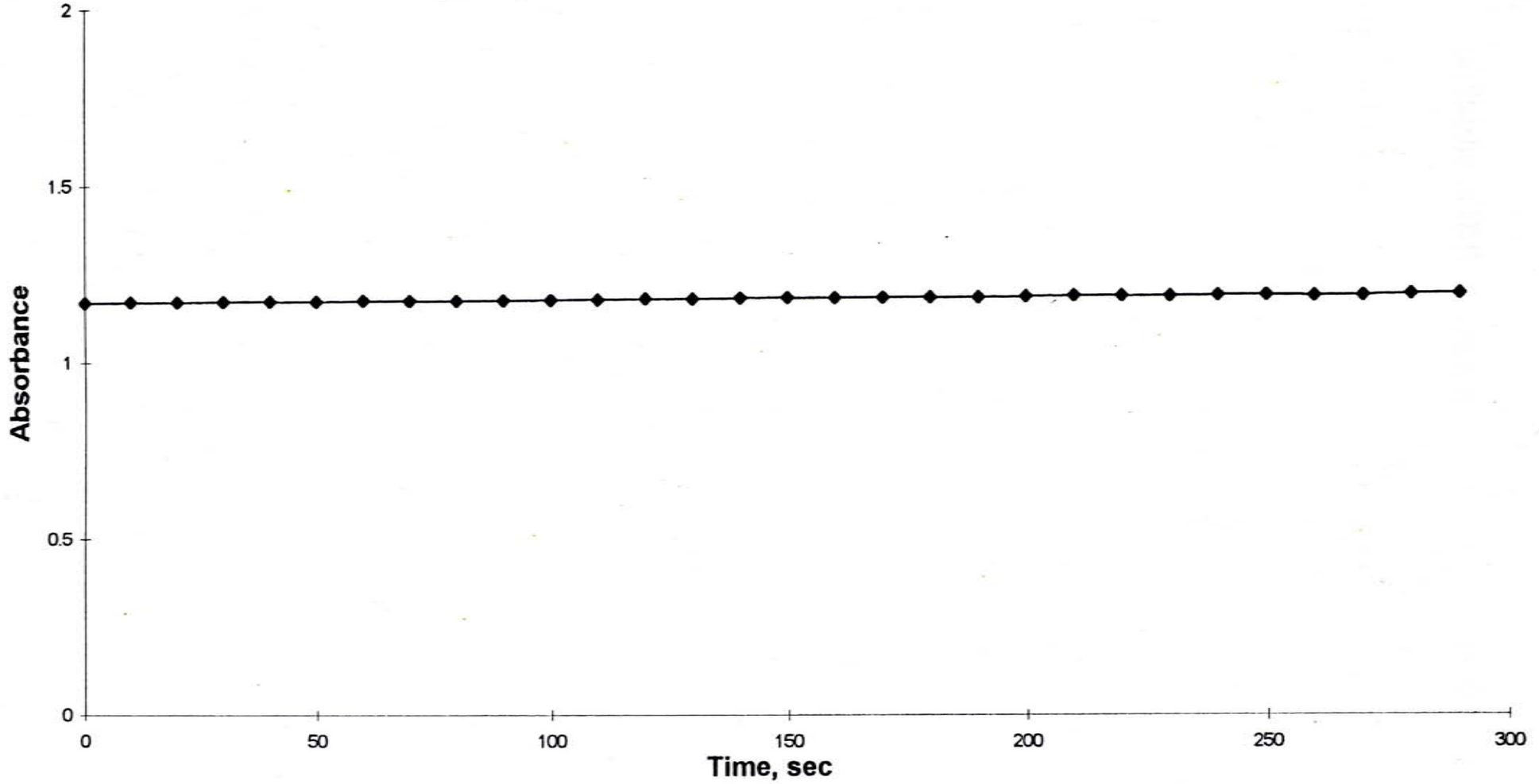


Figure 5.4 Stability of DMB solution

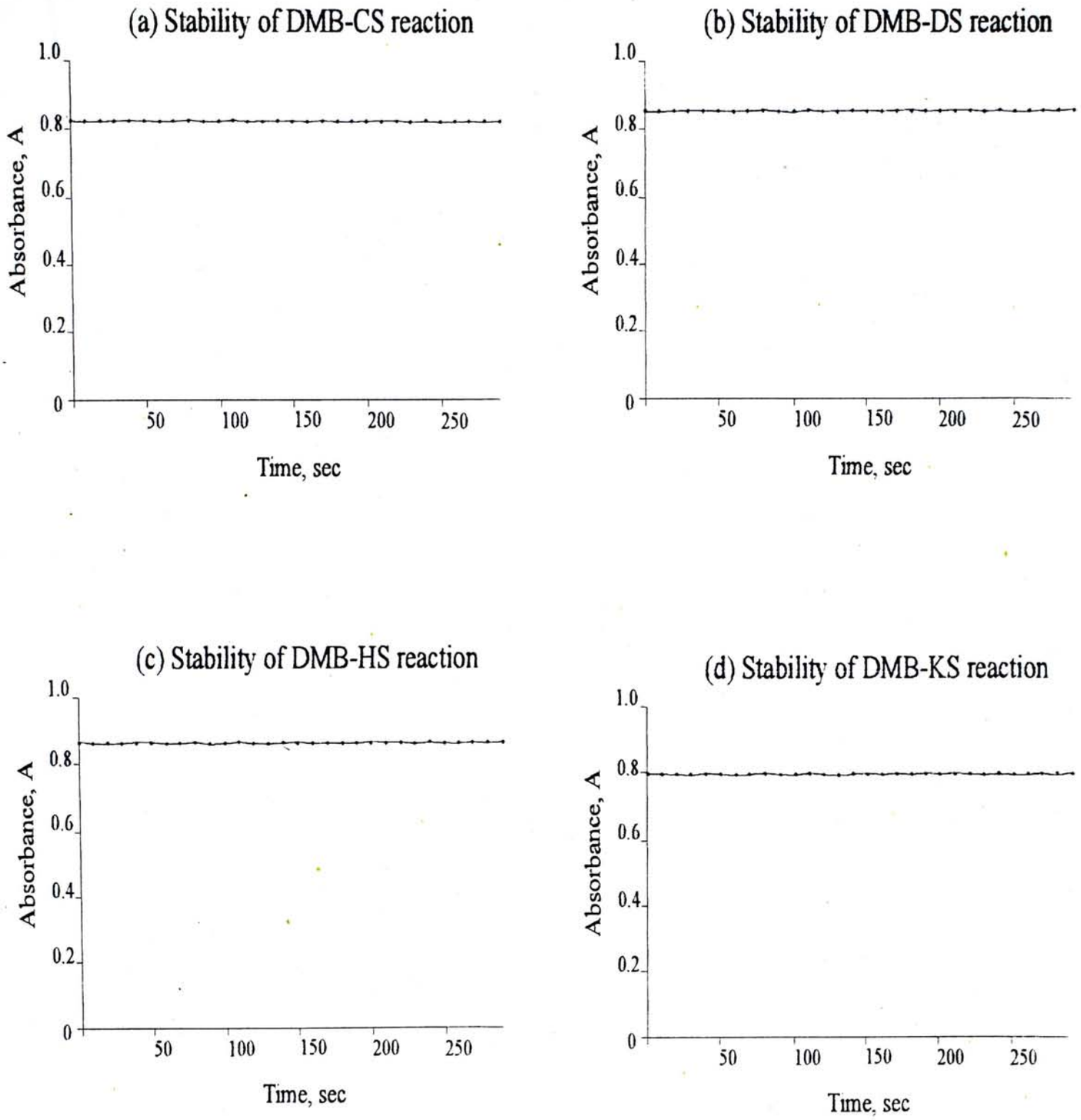


Figure 5.5 Stability of DMB-GAG reaction

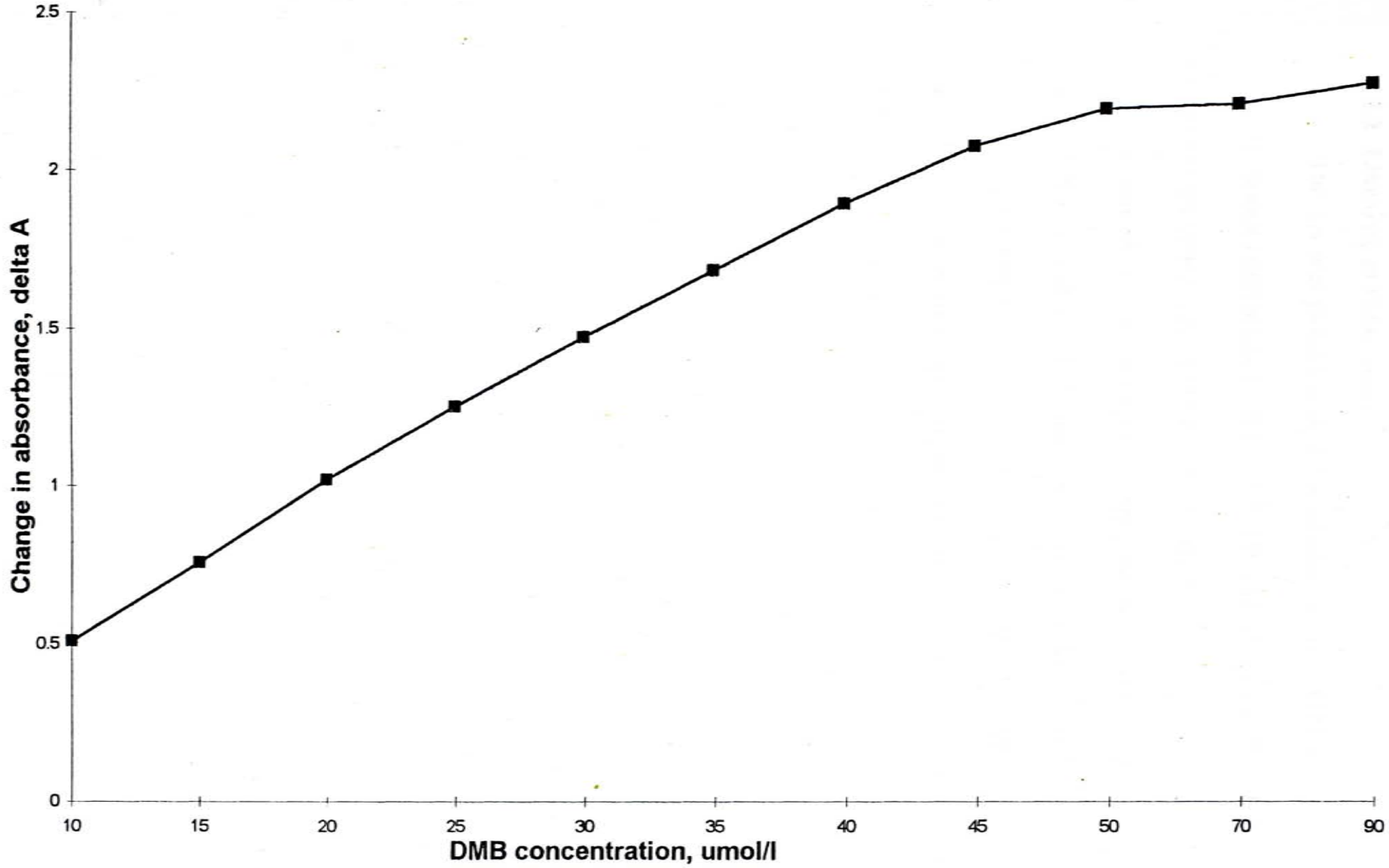


Figure 5.6 Linearity of DMB dye

5.3.2 Linearity of GAG assay

The ΔA was plotted against the concentration of GAGs. Figure 5.7 depicted ΔA against various concentrations of CS, DS, HS and KS. It was found that ΔA was linearly related to the concentration of CS, DS, HS and KS up to 70 mg/L. Figure 5.7 showed that the four linearity curves had similar slopes for GAG concentration up to 70mg/L. The slopes (table 5.2) of the four standard curves were calculated and significant difference were tested by ANOVA. The slopes were found to be not significantly different. Similarly the intercepts of the four standard curves were calculated and found to be not significantly different by ANOVA.

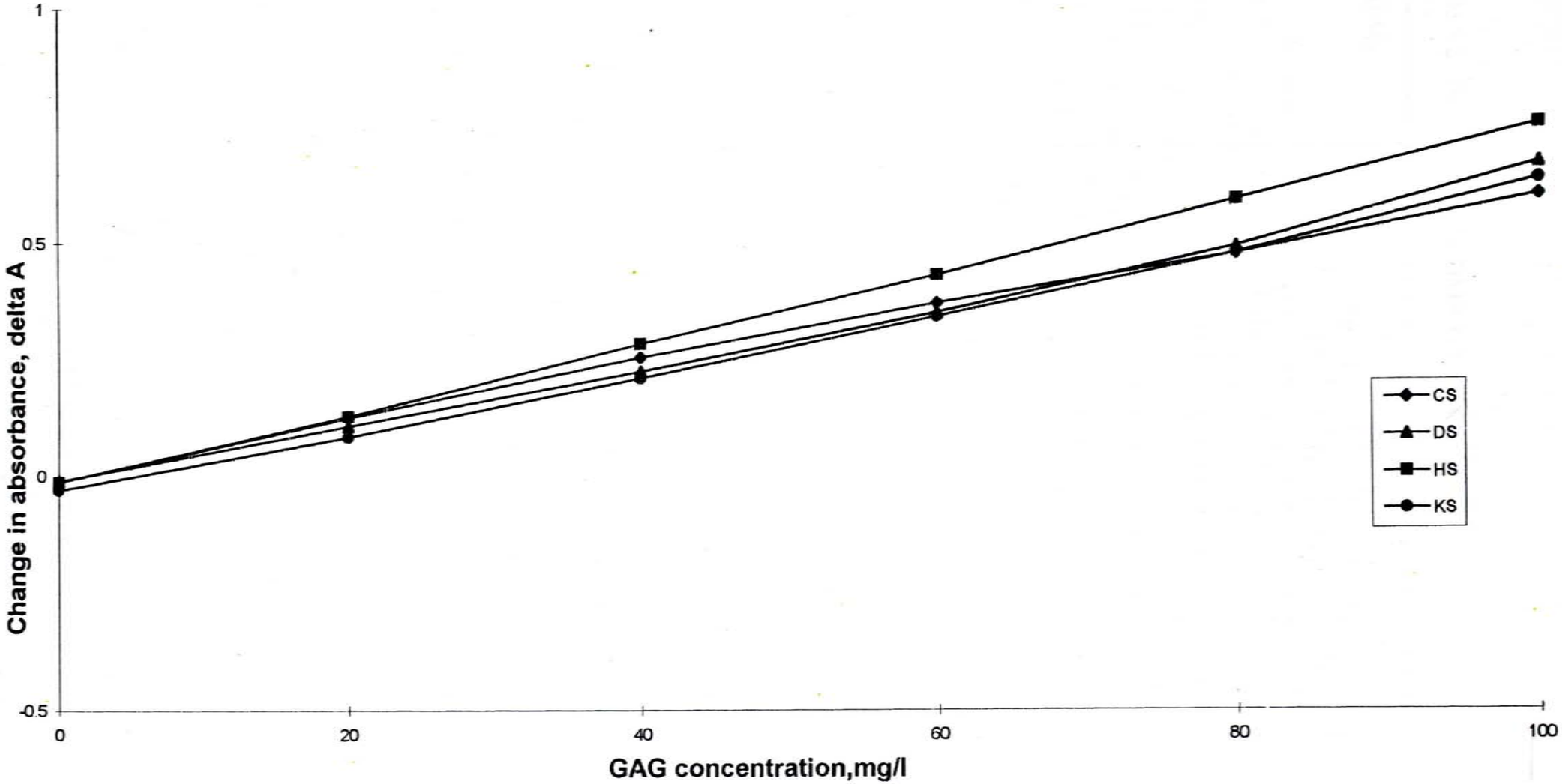


Figure 5.7 Reaction of DMB-GAG at various GAG concentration

Table 5.2 The slopes of the DMB-GAGs curves

GAG	Slope				
	1st run	2nd run	3rd run	4th run	5th run
CS	0.0102	0.0101	0.0102	0.0101	0.0099
DS	0.0101	0.0098	0.0106	0.0100	0.0093
HS	0.0103	0.0104	0.010	0.0101	0.0104
KS	0.0102	0.0102	0.0103	0.0103	0.0102

5.3.3 Detection limit

A blank sample was run 30 times. The mean ΔA was 0.103 and the standard deviation was 0.265. The corresponding lowest detection limit was read from the calibration curve and was found to equal to 0.8mg/L.

5.4 Precision study

The within-run and between-run precisions for the low and high controls were shown on table 5.3. The coefficient of variation was better than 7%.

Table 5.3 Result of precision study

	Within-run		Between-day	
Control level	Low	High	Low	High
mean (mg/L)	11.4	47.7	12.2	48.7
SD	0.5	1.1	0.8	1.3
CV(%)	4.5	2.4	6.5	2.6

5.5 Interference study

5.5.1 Haemoglobin

GAG concentration (mg/L) was measured in aqueous solutions of HS after addition of haemolysate. The haemoglobin concentrations varied from 0 to 5g/L. The results were shown in table 5.4. Haemoglobin did not interfere with the new DMB assay for concentration up to 5g/L.

5.5.2 Protein

GAG concentration (mg/L) was measured in aqueous solutions of HS after addition of serum bovine albumin. The protein concentrations varied from 0 to 5g/L. The result was shown in table 5.5. There was no interference with the new DMB assay by albumin concentration up to 5g/L.

5.6 Recovery study

The result was given in table 5.6. Concentration of the HS standard ranged from 10 to 50mg/L. Urinary GAG concentration was 8.4 mg/L. The mean percentage of recovery was 100.57%.

5.7 Correlation study

Results of the comparison between the new DMB method and the CPC/Carbazole reaction for forty urine samples were shown in figure 5.8. The present method correlated well with the CPC/Carbazole assay for GAG. Linear regression analysis gave a correlation coefficient(R) of 0.929. After Deeming's correction, the linear regression equation can be expressed as: [new DMB]= 0.87[CPC/Carbazole]+4.69

Table 5.4 Interference study by haemoglobin

	Measured HS Concentration(mg/L)					
Haemolysate concentration (g/dL)	0.0	0.1	0.2	0.3	0.4	0.5
sample 1	0.0	0.0	0.0	0.5	0.0	0.2
sample 2	11.2	10.8	11.0	11.1	11.0	10.7
sample 3	18.2	18.0	19.4	20.0	19.4	18.4
sample 4	40.2	40.1	39.9	40.2	40.1	41.6

Table 5.5 Interference study by protein

	Measured HS Concentration (mg/L)					
serum bovine albumin (g/L)	0	1	2	3	4	5
Sample 1	0.0	0.0	0.0	0.5	0.0	0.2
Sample 2	18.6	18.7	17.7	17.2	18.0	17.0
Sample 3	30.7	31.0	29.9	29.6	30.1	29.9
Sample 4	59.7	59.2	60.1	58.4	58.6	58.0

Table 5.6 Recovery study

	R_{base}	R_{test}		
Concentration in mg/L		10	25	50
first run	8.3	13.3	20.7	33.6
second run	8.4	13.4	20.9	33.9
third run	8.6	13.6	21.0	33.8
mean	8.4	13.4	20.9	33.7
% recovery		99.9	99.8	101.3

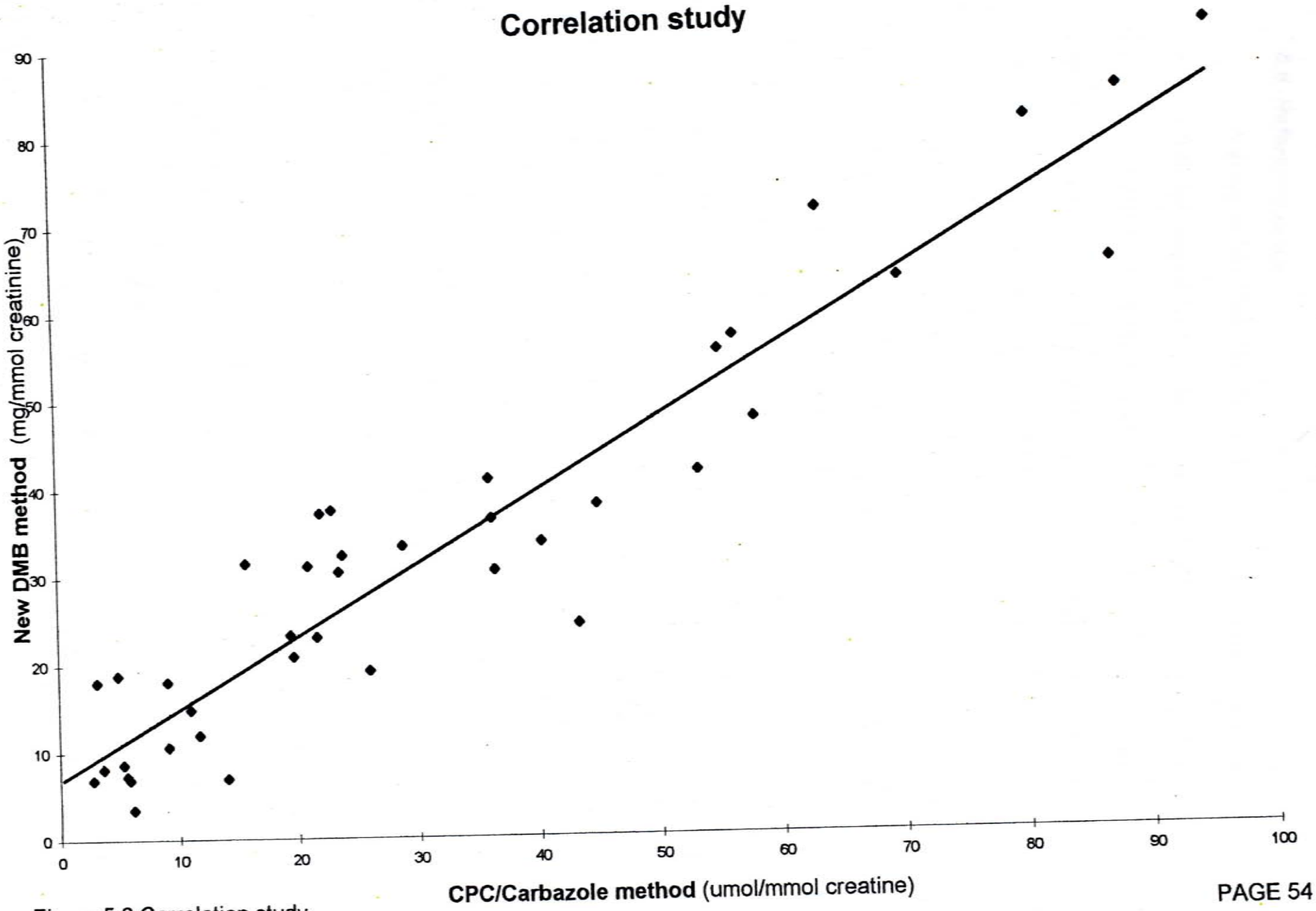


Figure 5.8 Correlation study

5.8 Reference range

A group of 546 (male 285, female 261) apparently healthy Hong Kong Chinese subjects with age ranging from 1 day to 70 years had been recruited in the reference population. Table 5.7 gave the reference value of urinary GAG/creatinine ratio as a function of age and sex. The GAG/creatinine ratio was not significantly different between the sexes (stendent t-test, $P>0.05$).

The reference values for the new DMB assay were age dependent (figure 5.9). The change in GAG as a function of age was greatest in young age groups. In those older than 20 years, GAG content was nearly constant. In view the non-Gaussain distribution in each age-group, non-parametric method was used to derive the reference ranges(table 5.8).

5.9 GAG in mentally retarded patients

Urine samples from 295 mentally retarded patients living in Caritas Medical center were collected. Urine GAG was measured and the result was expressed in amount of GAG in relation to urine creatinine excretion. Figure 5.10 showed the GAG excretion against age. 35% of these patients had GAG/creatinine above the 95% upper interval of the general population at the same age group($P<0.05$, Mann Whitney test). One of the samples had GAG content over 70mg/mmol creatinine.

Table 5.7 Reference values of urinary GAG/creatinine ratio measured by the new DMB assay according to sex and age

	Male			Female		
Age	N	Median mg/mmol	Range mg/mmol	N	Median mg/mmol	Range mg/mmol
0—5 months	50	37.8	23.1-50.5	70	38.0	24.1-52.1
6—12 months	9	18.9	15.9-22.5	12	20.1	16.5-24.0
13—23 months	12	18.7	14.8-22.1	14	18.5	15.9-22.2
2—3 years	15	17.7	12.1-22.1	16	17.5	12.3-21.9
4—5 years	26	14.8	10.5-19.7	27	15.1	10.8-19.5
6—7 years	18	13.7	10.2-16.0	14	13.9	10.1-16.9
8—9 years	11	11.8	9.1-13.2	18	12.0	9.6-13.5
10—14 years	40	5.5	2.9-8.1	37	6.9	3.5-9.1
15—19 years	51	3.1	1.2-5.0	42	2.9	1.5-4.9
>20 years	48	2.7	0.2-4.8	54	2.2	0.2-4.6

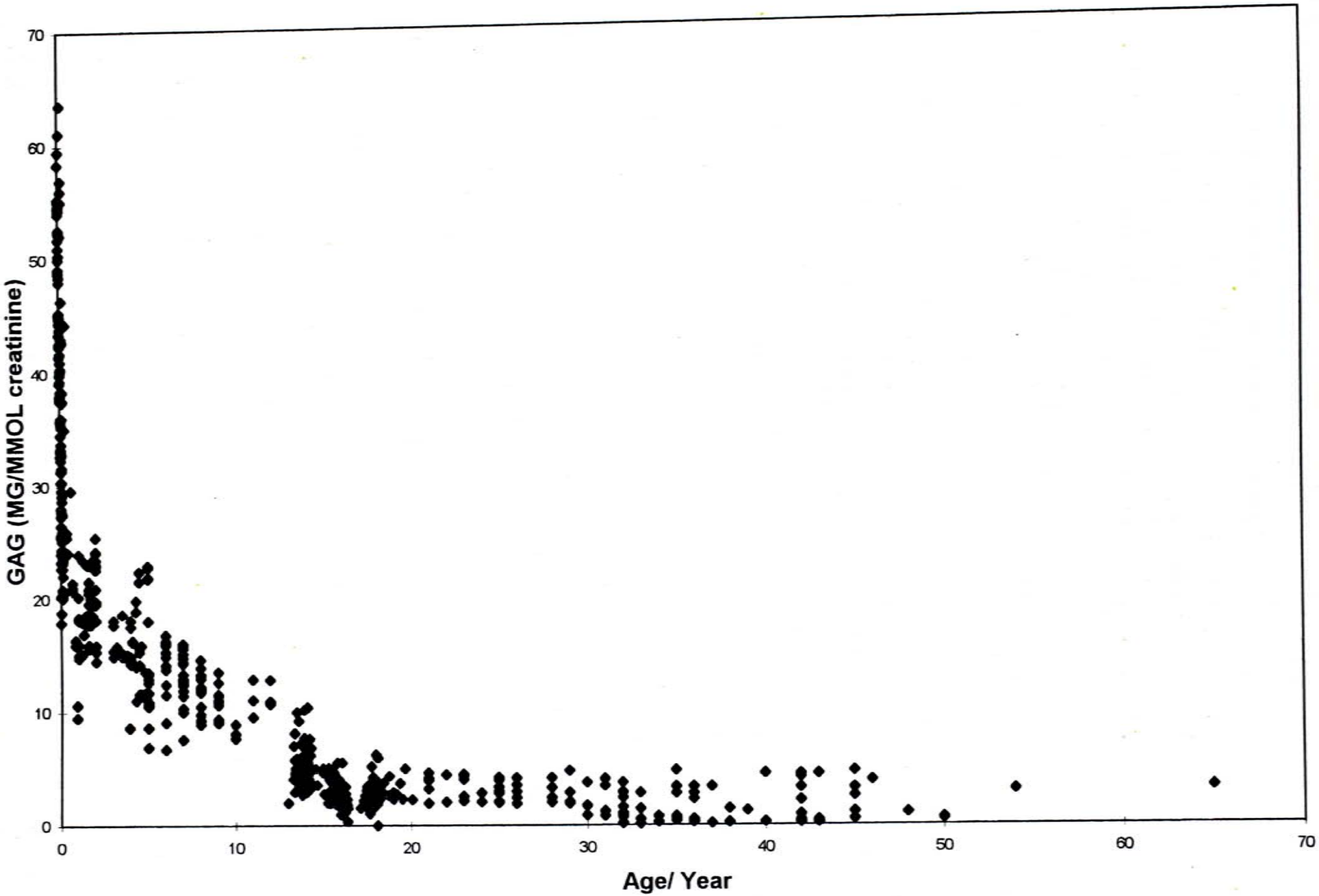


Figure 5.9 GAG content in urine samples from reference population

Table 5.8 Reference range of urine GAG/creatinine ratio by non-parametric method (2.5 th percentile to 97.5 th percentile)

Age	GAG/creatinine mg/mmol creatinine (2.5^{th}-97.5^{th} percentile)
0—5 months	20.8—56.0
6—12 months	15.9—24.5
13—23 months	15.6—21.1
2—3 years	14.6—20.6
4—5 years	10.5—20.0
6—7 years	9.1—16.1
8—9 years	9.0—13.5
10—14 years	3.3—10.7
15—19 years	1.4—5.2
>20 years	0.8—4.5

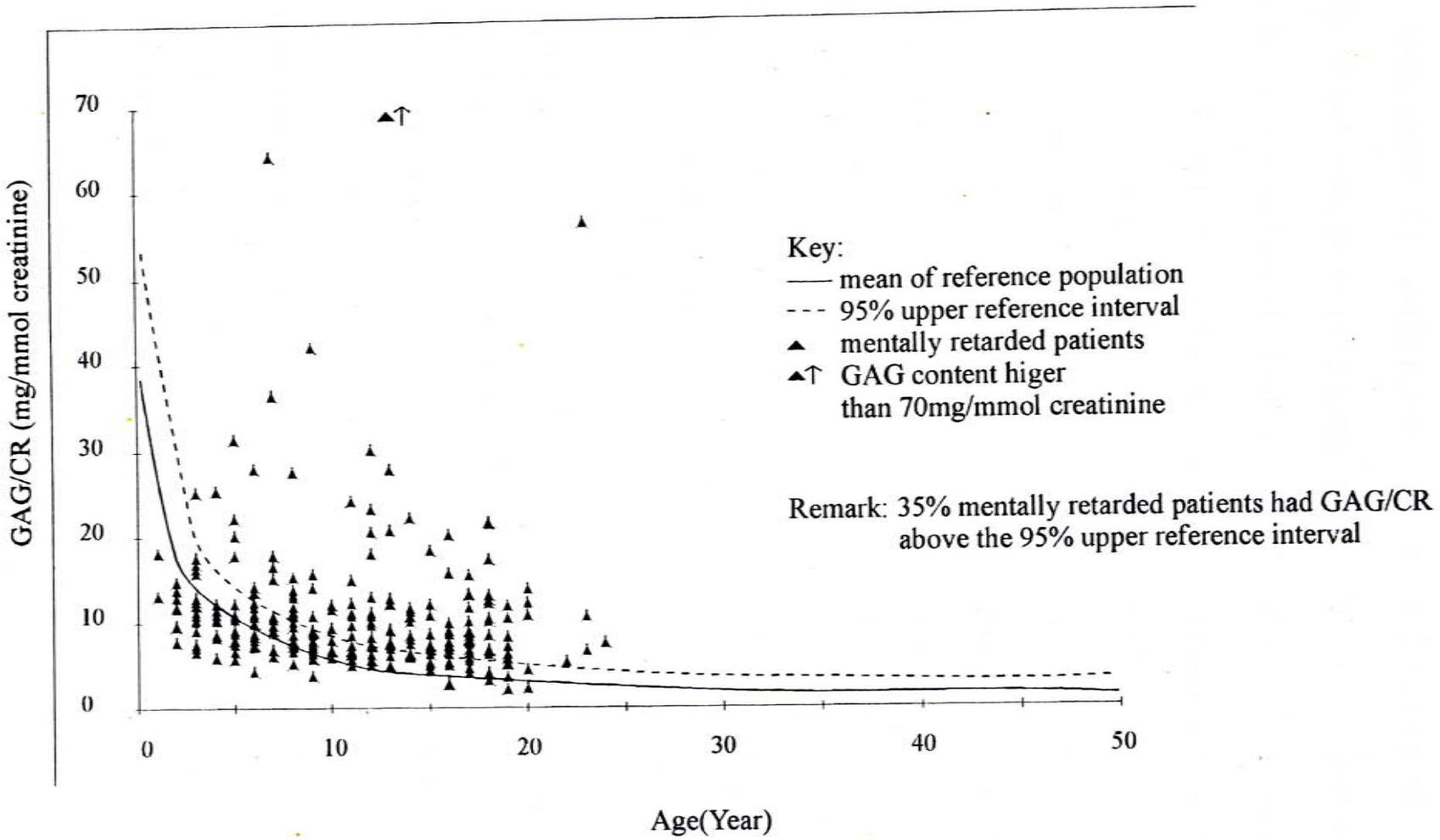


Figure 5.10 GAG content in mentally retarded patients

6. Discussion

Some degree of successful treatment of MPS has been achieved recently(19, 20, 22). An efficient, low-cost screening method for MPS becomes essential. In recent years, direct dye binding methods for GAG quantitation appeared in the literature, such as AB(50), DMB(1,51), DMB-TRIS(49) methods. They provided a direct quantitation of urinary GAGs, doing away with the precipitation step. Obviously these methods were simpler to perform and faster to obtain results. In the evaluation of the analytical performance of AB and DMB, de Jong et al (49) found that DMB had a higher sensitivity than AB in the differentiation of normal persons from MPS patients. They concluded that DMB assay was a better screening method for MPS than AB assay.

The DMB assays reported in the literature monitored the increase of absorbance of the DMB-GAG complexes at about 530nm. The spectral characteristics of different DMB-GAG complexes were investigated in this study. The absorption maxima of the complexes were found to vary between 528-541nm. Similar finding was obtained by Stone et al (4). Measurement of products at one wavelength would lead to inaccurate results depending on the relative composition of different GAGs in the samples. The measurement of the complexes at 530nm would also have spectral interference by haemoglobin. However the spectrum of the pure DMB dye had two peaks: 593 and 649 nm. An alternative solution would be to measure the decrease in absorbance at either one of the peak wavelengths of the pure dye. This was the basic principle of the established new DMB method in this project.

The DMB-DS reaction at the three absorption peaks were investigated. The decrease in magnitude of absorbance of the pure dye at 647nm was greater than that of the increase in absorbance of the complex at 520nm. But the response at wavelength 593nm was even higher. These observations agreed with the findings by Stone et al (4).

The original DMB assay used formic acid as buffer(3). However it was found to have interference by urinary protein and haemoglobin(67). An improved DMB assay was developed by de Jong (6) which involved the use of a tris-buffer. Such modification was claimed to remove the interference by protein. Interference by haemoglobin could be removed by including a blank measurement.

This study attempted to measure GAGs at 593nm using a tris buffer. The DMB pure dye and the reaction complexes of DMB-GAG were found to be stable for up to 300s. There were no significant difference in reactivity of the four GAGs towards the DMB dye. This observation agreed with the finding by Whitley et al(3).

6.1 Analytical Performance

The new DMB assay was automated in a Cobas Bio autoanalyzer. The sample volume used in this assay was 20µl. The response of the reaction was monitored at wavelength 593 for 40s. The lowest detection limit was 0.8mg/L and the linearity range was found to be up to 70mg/L using a DMB concentration of 35 µmol/L. Sensitivity analysis showed that this method gave an absorbance change of 0.285A per µg of GAG in the reaction cell as compared to de Jong et al (49) 0.242A per µg and Whitley et al (3) 0.05A per ug. Hence, this new method was more sensitive and was expected to have improved precision. Albumin and haemoglobin did not interfere with this assay up to 5g/L and 5g/L respectively. These findings agreed with those by de Jong et al(6).

The new DMB assay was compared with the conventional CPC/Carbazole method. Deeming's correction was applied for the correlation study to take account of the assay uncertainty of the CPC/Carbazole method. The correlation coefficient was 0.929 and the linear regression equation was [new DMB]= 0.87[CPC/Carbazole]+4.69. Similar finding was obtained by a study(66) which showed that the DMB assay correlated well with the CPC/Carbazole test(r=0.837).

The new DMB method gave more precise results than the conventional method. Within-run imprecisions were 4.5% and 2.4% at 11 and 48 mg/L respectively. Between-batch imprecisions were 6.5% and 2.6% at 11 and 48 mg/L respectively. The CPC/Carbazole method usually had a precision of over 10% at these levels. Similar findings were obtained a study by de Jong et al(6). The imprecision of their DMB assay varied from 2% to 7% at GAG between 22-72mg/L. This study demonstrated a recovery

from 99% to 101%. It was better than anther study(66), which showed a recovery of 71% to 107%.

The new DMB method was simple and fast to measure the urinary GAG. It required only about 5 minute to assay a batch of 24 samples. Small sample volume (20μl in this assay) was certainly an advantage to screen samples from neonates. The CPC/Carbazole method required large sample volumes (2-10ml urine) which could not be routinely collected from infants. It could be automated in other modern automatic chemistry analyzer. However, in many modern chemical analyzers, discrete filters were usually employed. They might not have a filter for 593nm. In view of the greater response at 593nm as seen in this study, a nearby wavelength could be used instead without greatly affecting the performance of the assay.

6.2 *Clinical performance*

6.2.1 Reference population

Urine for analysis could be collected as a 24-hour urine sample or as a random urine. 24-hour urine samples were not convenient and incomplete collection could lead to erroneous result. Spot urine samples were used because it was easy to collect and could be obtained any time from the subjects especially infants. The GAGs content in the untimed urine samples were measured and expressed in relation to the creatinine excretion. Although creatinine excretion varied during a 24-hour period and the GAG/creatinine ratio was increased at night(7), both substances were excreted at fairly constant rates during the day(0800 to 1800). The GAG/creatinine ratios in samples obtained during this period agree reasonably well with those obtained on complete 24-hour collections(5). Infected samples should not be used (5). Fresh urine was more appropriate. If analysis could not be performed as soon as possible, the samples were best frozen after collection and stored at -70°C.

This study measured spot urine GAG-creatinine ratio from 546 apparently healthy Hong Kong Chinese subjects with age ranging from 1 day to 70 years. The reference values for the new DMB assay were, as for other DMB assays(5,6), age dependent. The GAG/creatinine ratio was not significantly different between the sexes($P>0.05$, student t-test). The GAG excretion was high in the neonatal period because of the rapid growth rate at this time of life. The GAG excretion then decreased with age and leveled off after age 20. This age dependent reference range was found to be similar to the findings by de Jong et al (6). No previous reference range for Chinese subjects was reported in the literature.

This was the first established reference range for Hong Kong Chinese population. However, the established reference range in this study was similar to other group of studies from western countries(5,6). Reference range from western population could be applied to normal Chinese subjects.

6.2.2 Mentally retarded patients

295 subjects were selected from a group of mentally retarded patients. These patients were living in a care center for the handicapped, mentally retarded patients. 35% of these patients had GAG/creatinine above the 95% upper interval of the general population at the same age group($P<0.05$, Mann Whitney test). Some of these patients and very high GAG/creatinine ratio eg. 70mg/mmol creatinine and they may be undiagnosed MPS patients. Confirmation methods such as two dimensional electrophoresis or enzyme techniques should be carried out to evaluate these cases.

This group of patients is associated with a failure in physical development. In these patients delayed growth or altered metabolism may be present. Their physical development(eg. bone, cartilage) could be lagged behind their chronological age. Since GAG/creatinine ratio is age dependent, it might explain why there were 35% of these patients had elevated GAG excretion above the upper reference interval. Probably a separate reference range should be established for these patients. Elevated GAG excretion was also found in patients with connective tissue destruction (5). The mentally retarded patients recruited in this study might also have these disorders that caused a significant higher GAG/creatinine ratio. These results also suggested that there is a need to investigate the cause of mentally retarded patients in Hong Kong.

6.2.3 Suggestion for future studies

The new DMB assay was only screening test. Elevated results should be confirmed by enzymatic technique. The assay was only linear up to 70mg/L. Abnormally high results required dilution. Interference from other substances such as drugs, coloured dyes or abnormal metabolites that commonly presented in urine had not been studied.

In the present study, only 546 normal subjects were recruited for establishing the reference range. More reference subjects at different age group could be recruited. They were only checked for renal and liver diseases. Many disorders were known to cause elevated urinary GAG, such as rickets, malabsorption syndromes, osteomalacia, leukaemia, disseminated lupus erythematosus, rheumatoid arthritis and some rare disorders(5). These abnomalities should be considered in future recruitment of reference subjects. Nevertheless, the established reference range was similar to those reported in the literature.

Mentally retarded patients were shown to have elevated GAG excretion in this study. The causes of such elevation should attract more investigations to see whether a separated reference range is needed to be established for the patients in the care home.

7. Conclusion

The new DMB assay was simple and precise for quantitation of GAG contents in urine samples. It was found to be free from interference by protein and haemoglobin. Good correlation was found with the CPC/Carbazole method. It gave a higher response than the original DMB assay or the DMB-tris assay.

This assay can be automated in any modern analyzer. GAG/creatinine ratio decreased significantly with age. But no significantly difference between the sexes was found. The sample volume was as little as 20ul, making it possible for screening neonates. Mentally retarded patients were shown to have higher GAG content than the general population at the same age groups. Further evaluation should be carried out to investigate why there was such discrepancy.

8. Appendix

Appendix 1 Instrument settings for DMB stability study:

	-MUCO	
1.	Units	MG/L
2.	Calculation factor	1
3.	Standard 1 concentration	0
4.	Standard 2 concentration	0
5.	Standard 3 concentration	0
6.	Limit	0
7.	Temperature (^{O}C)	25
8.	Type of analysis	1
9.	Wavelength (nm)	593
10	Sample volume (μl)	0
11	Diluent volume (μl)	0
12	Reagent volume (μl)	250
13	Incubation time (seconds)	0
14	Start reagent volume (μl)	0
15	Time of first reading (seconds)	0.5
16	Time interval (seconds)	10
17	Number of readings	30
18	Blanking mode	0
19	Printout mode	3

Appendix 2 Instrument settings for DMB linearity study:

	-MUCO	
1.	Units	MG/L
2.	Calculation factor	1
3.	Standard 1 concentration	0
4.	Standard 2 concentration	0
5.	Standard 3 concentration	0
6.	Limit	0
7.	Temperature (^{O}C)	25
8.	Type of analysis	1
9.	Wavelength (nm)	593
10	Sample volume (μl)	0
11	Diluent volume (μl)	0
12	Reagent volume (μl)	250
13	Incubation time (seconds)	10
14	Start reagent volume (μl)	0
15	Time of first reading (seconds)	0.5
16	Time interval (seconds)	40
17	Number of readings	2
18	Blanking mode	0
19	Printout mode	5

9. Reference

1. de Jong JGN, Wevers RA, Laarakkers C and Poorthuis BJHM. Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine: a rapid screening procedure for mucopolysaccharidoses. *Clin Chem* 1989;35:1472-7.
2. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986;883:173-7.
3. Whitley CB et al. Diagnostic test for Mucopolysaccharidoses, I. Direct method for quantifying excessive urinary glycosaminoglycan excretion. *Clin Chem* 1989;35:374-9.
4. Stone JE, Akhtar N, Botchway S and Pennock CA. Interaction of 1,9-dimethylmethylene blue with glycosaminoglycans. *Ann Clin Biochem* 1994;31:147-52.
5. Pennock CA. A review and selection of simple laboratory methods used for the study of glycosaminoglycan excretion and the diagnosis of the mucopolysaccharidoses. *J Clin Path* 1976;29:111-23.
6. de Jong JGN et al. Measuring urinary glycosaminoglycans in the presence of protein: An improved screening procedure for Mucopolysaccharidoses based on dimethylmethylene blue. *Clin Chem* 1992;38:803-7.
7. Sly WS. The mucopolysaccharidoses, Chapter 10. In: Metabolic Control and Disease, Philadelphia, Saunders 1980, p545-81.
8. Spranger J. The mucopolysaccharidoses. In: Emery AEH et al, eds. Principles and practice of medical genetics. New York: Churchill Livingstone, 1983:1339-47.
9. Neufeld EF and Muenzer J. The mucopolysaccharidoses, Chapter 61. In: The metabolic basis of inherited disease, ed 6, McGraw Hill 1989, p1565-87.

10. Hunter C. A rare disease in two brothers. *Proc R Soc Med* 1917;10:104-15.

11. Hurler G. Ueber einen Type multipler Abortungen, vorwiegend am Skelett System. *Z Kinderheilk* 1919;24:220-34.

12. Brante G. Gargoylism: a mucopolysaccharidosis. *Scand J Clin Laboratory Invest* 1952;4:43-6.

13. Dorfman A and Lorinez AE. Occurrence of acid mucopolysaccharides in the Hurler syndrome. *Proc Natl Acad Sci* 1957;43:443-6.

14. McKusick VA. Heritable disorders of connective tissue, ed 3. St Louis, CV Mosby Co, 1966.

15. McKusick VA and Neufeld EF. The mucopolysaccharide storage diseases, Chapter 36. In: The metabolic basis of inherited disease, ed 5, McGraw Hill 1983, p751.

16. Dean MF et al. Enzyme replacement therapy by fibroblast transplantation. *J Clin Invest* 1979;63:138-46.

17. Adinolfi M, Brown S. Strategies for the correction of enzymatic deficiencies in patients with mucopolysaccharidoses. *Dev Med Child Neurol* 1984;26:404-8.

18. Hugh-Jones K. Psychomotor development of children with mucopolysaccharidoses type I-H following bone marrow transplantation. *Birth Defects* 1986;22:25-9.

19. Whitley CB et al. Bone marrow transplantation for Hurler syndrome: Assessment of metabolic correction. *Birth Defects* 1986;22:7-24.

20. Hobbs JR, Hugh-Jones K, Barrett AJ et al. Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone marrow transplantation. *Lancet* 1981;ii:709-12.

21. Warkentin PI et al. Bone marrow transplantation in Hunter syndrome: A preliminary report. *Birth Defects* 1986;22:31-9.

22. Krivit W et al. Bone marrow transplantation in the Maroteaux-Lamy syndrome (mucopolysaccharidoses type VI). *N Engl J Med* 1984;311:1606-11.

23. McGovern MM et al. Status of bone marrow transplantation in the Maroteaux-Lamy syndrome(MPS type 6): Status 40 months after BMT. *Birth Defects* 1986;22:41-53.

24. Van de Kamp, Niermeijer MF, Von Figura K and Giesberts M. Genetic Heterogeneity and clinical variability in the Sanfilippo syndrome(type A, B and C). *Clin Genet* 1981;20:152-60.

25. Lowry RB, Renwick DHG. Relative frequency of the Hurler and Hunter syndromes. *N Engl J Med* 1971;284:221-2.

26. Schaap T, Bach G. Incidence of mucopolysaccharidoses in Israel: Is Hunter disease a 'Jewish disease'? *Hum Genet* 1980;56:221-3.

27. Van De Kamp. The Sanfilippo syndrome: a clinical and genetical study of 75 patients in the Netherlands. Doctoral thesis. 'S-Gravenhage, JH Pasmans, 1979.

28. Muenzer J. Mucopolysaccharidoses. *Adv Pediatr* 1986;33:269-302.

29. Lee JES, Falk RE, Ng WG, Donnell GN. β-Glucuronidase deficiency: A heterogeneous mucopolysaccharidoses. *Am J Dis Child* 1985;139:57-9.

30. Berry HK and Spinanger J. A paper spot test useful in study of Hurler's syndrome. *J Laboratory Clin Med* 1960;55:136-8.

31. Carson NAJ and Neill DW. Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland. *Arch Dis Childh* 1962;37:505-13.

32. Procopsis PG, Turner B, Ruxton JT and Brown DA. Screening tests for mucopolysaccharidoses. *J Ment Defic Res* 1968;12:13-7.

33. Berman ER, Vered J and Bach G. A reliable spot test for mucopolysaccharidoses. *Clin Chem* 1971;17:886-90.

34. Dorfman A. Studies on the biochemistry of connective tissue. *Pediatrics* 1958;22:576-89.

35. Denny W and Dutton G. Simple urine test for gargoylism. *Brit Med J* 1962;1:1555.

36. Renuart AW. Screening for inborn errors of metabolism associated with mental deficiency or neurological disorders or both. *New Engl J Med* 1966;274:384-7.

37. Manley G and Hawksworth J. Diagnosis of Hurler's syndrome in the hospital laboratory and the determination of its genetic type. *Arch Dis Childh* 1966;41:91-6.

38. Pennock CA. A modified screening test for GAG excretion. *J Clin Path* 1969;22:379-80.

39. Scott JE. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. *Meth Biochem Anal* 1960;8:145-97.

40. Segni G, Romano C and Tortorolo G. Diagnostic test for gargoylism. (Letter) *Lancet* 1964;2:420.

41. Rosenfeld L. Quantitative levels of the constituents of acid mucopolysaccharides and other carbohydrate polymers in dialyzed normal human urine. *Clin Chim Acta* 1971;31:263-9.

42. Wessler E. Determination of acidic glycosaminoglycan (mucopolysaccharides) in urine by an ion exchange method: Application to 'collagenoses', gargoylism, Nail-Patella Syndrome and Farber's disease. *Clin Chim Acta* 1967;16:235-43.

43. Blackham GA and Raine DN. The biochemistry of the mucopolysaccharidoses. *Ann Clin Biochem* 1970;6:49-54.

44. Thompson GR and Castor CW. The excretion of nondialyzable urinary mucopolysaccharide in rheumatic and other systemic disease states. *J Laboratory Clin Med* 1966;68:617-27.

45. Teller WM, Burke EC, Rosevear JW and McKenzie BF. Urinary excretion of acid mucopolysaccharides in normal children and patients with gargoylism. *J Laboratory Clin Med* 1962;59:95-101.

46. Bitter T and Muir HM. A modified uronic acid carbazole reaction. *Ann Biochem* 1962;4:330-4.

47. Dean MF, Muir H and Ewins RJF. Hurler's, Hunter's and Morquio's syndromes. A biochemical study in the light of current views of the underlying defects. *Biochem J* 1971;123:883-94.

48. Dische Z. A new specific colour reaction of hexuronic acids. *J Biol Chem* 1947:167;189-98.

49. de Jong JGN, Heijs WM and Wevers RA. Mucopolysaccharidoses screening: Dimethylmethylene blue versus alcian blue. *Ann Clin Biochem* 1994;31:267-71.

50. Gold EW. A simple spectrophotometric method for estimating glycosaminoglycan concentrations. *Anal Biochem* 1979;99:183-8.

51. Farndale RW, Sayers CA and Barrett AJ. A direct spectrophotometric microassay for sulphated glycosaminoglycans in cartilage cultures. *Connect Tiss Res* 1982;9:247-8.

52. Gold EW. The quantitative spectrophotometric estimation of total sulphated glycosaminoglycan levels: Formation of soluble alcian blue complexes. *Biochim Biophys Acta* 1981;673:408-15.

53. Hall CW, Liebaers I, Di Natale P and Neufeld EF. Enzymatic diagnosis of the genetic mucopolysaccharide storage disorders. *Methods Enzymol* 1978;50:439-56.

54. Kresse H, Von Figura K et al. Enzymatic diagnosis of the genetic mucopolysaccharide storage disorders. *Methods Enzymol* 1982;83:559-72.

55. Migeon BR, Sprenkle JA Liebaers I et al. X-linked Hunter syndrome: The heterozygous phenotype in cell culture. *Am J Hum Genet* 1977;29:448-54.

56. Tonnesen T, Goultler F, Lykkelund C. Reliability of the use of fructose 1-phosphate to detect Hunter cells in fibroblast cultures of obligate carriers of the Hunter syndrome. *Hum Genet* 1983;64:371-5.

57. Nwokoro N and Neufeld EF. Detection of Hunter heterozygotes by enzymatic analysis of hair roots. *Am J Hum Genet* 1979;31:42-9.

58. Yutaka T, Fluharty AL, Stevens RL et al. Iduronate sulfatase analysis of hair roots for identification of Hunter syndrome heterozygotes. *Am J Hum Genet* 1978;30:575-82.

59. Wessler E. Analytical and preparative separation of acidic glycosaminoglycans by electrophoresis in barium acetate. *Anal Biochem* 1968;26:439-44.

60. Lewis PW, Raine DN and Kennedy JF. Recognition of the mucopolysaccharidoses by four screening tests, including a refinement of the albumin turbidity test and their differentiation by electrophoretic separation of urinary glycosaminoglycans. *Ann Clin Biochem* 1974;11:67-71.

61. Wusteman FS, Lloyd AG and Dodgson KS. Thin-layer chromatography and the rapid identification of common acidic glycosaminoglycans. *J Chromatog* 1966;21:32-9.

62. Teller WM and Ziemann A. Thin layer chromatography of urinary acid glycosaminoglycans, a screening procedure for mucopolysaccharidoses. *Hormone Metab Res* 1969;1:32-5.

63. Humbel R and Chamoles NA. Sequential thin layer chromatography of urinary acid glycosaminoglycans. *Clin Chim Acta* 1972;40:290-3.

64. Lippiello L and Mankin HJ. Thin layer chromatographic separation of the isomeric chondroitin sulphates, dermatan sulphate and keratan sulphate. *Analyt Biochem* 1971;39:54-8.

65. Kodama C, Ototani N, Isemura M, Aikawa J, Yosizawa Z. Liquid-chromatographic determination of urinary glycosaminoglycans for differential diagnosis of genetic mucopolysaccharidoses. *Clin Chem* 1986;32:30-4.

66. Panin G, Naia S et al. Simple spectrophotometric quantification of urinary excretion of glycosaminoglycan sulphates. *Clin Chem* 1986;32:2073-6.

67. Piraud M, Maire I, Mathieu M. Pitfalls of screening for Mucopolysaccharidoses by the dimethylmethylene blue test. *Clin Chem* 1993;39:163.